THE EFFECTS OF ASBESTOS INJECTION ON THE PERITONEAL MACROPHAGE POPULATION OF THE MOUSE

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I declare that this thesis was composed by myself and that the work contained in it is my own.

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A B S T R A C T

ABSTRACT

Asbestos is a naturally occurring fibrous silicate mineral which has extensive industrial and domestic application: exposure to asbestos is associated with pulmonary fibrosis, lung cancer and mesothelioma. Following inhalation of asbestos the fibres are rapidly taken up by alveolar macrophages and macrophages have been implicated in development of the asbestos related pulmonary diseases. In this thesis macrophages harvested from the peritoneal cavity of mice following intra-peritoneal injection of asbestos (UICC samples) were used as convenient models to study the effect of asbestos on macrophages in vivo with particular reference to membrane characteristics.

Macrophages harvested from the peritoneal cavity 3-70 days after injection of asbestos were found to be activated by several criteria. Latex spheres and saline, injected as controls, failed to induce activated macrophages. An in vitro nucleotide release assay using syngeneic fibrosarcoma cells as targets revealed that asbestos activated macrophages were negligibly cytotoxic to tumour cells. Supernatants from asbestos activated macrophages were not cytostatic to fibrosarcoma cells in vitro. The results from the in vitro assays were in general confirmed by in vivo assays for tumour growth modulating effects of asbestos. Thus intraperitoneal injection of asbestos into animals with concomitant experimental subcutaneous fibrosarcomas in the main had no significant effect on tumour growth although an intermediate dose (5 mg) did cause small significant reductions in tumour growth.

Supernatants from asbestos activated macrophages suppressed thymocyte mitogenesis, by Concanavalin A, by up to 50% but did not affect proliferation of fibroblasts. Latex and saline induced macrophage supernatants had no suppressive activity.

In studies on the membrane of asbestos activated macrophages Concanavalin A was used as a probe for membrane glycoprotein. Concanavalin A was used to visualise its specific receptor glycoprotein in the membrane of macrophages interacting with asbestos using both light microscopy and electron microscopy; some rearrangement of Concanavalin A receptor glycoprotein was observed. Incubation of asbestos activated macrophages with fluoresceinated Concanavalin A resulted in aggregation of the Concanavalin A receptor glycoprotein into caps in approximately one third of asbestos induced macrophages; this phenomenon was microfilament mediated. Radioactively labelled Concanavalin A showed that absolute numbers of Concanavalin A binding sites was not a major factor in this capping phenomenon.

In attempts to detect differences in surface antigenicity between asbestos activated and control macrophages, anti-sera were raised in rabbits and an $[^{125}I]$ Protein A binding assay was utilised. Absorption of activity against non-specific mouse antigens, however, left a very small specific anti-macrophage component and so differences could not be detected; this was confirmed by fluorescence and complement mediated lysis assays. An $[^{125}I]$ Protein A assay was also used to try and detect autoimmune deposition of antibody on to asbestos activated macrophages in vivo. There was no evidence of increased levels of $[^{125}I]$ Protein A binding to macrophages from dusted animals in either the short or long term.

Limited studies were carried out on malignant cell lines derived from peritoneal cells harvested from mice which had been injected with asbestos and kept to study the long term effects of asbestos in the model.

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INTRODUCTION

1.1 Asbestos: history and uses

Asbestos is a fibrous silicate mineral occurring in areas of the earth's crust, it can be spun and woven into textile which has good insulating properties, very large surface areas and is resistant to attack from weathering and chemicals. These unique properties of asbestos assured that even in early history, in the pre-minimg era when asbestos was encountered in surface strata, its properties were recognised and recorded. Thus articles made from a substance which was unmistakably asbestos were mentioned in the writings of Theophrastus (372-287 BC), Plutarch (64-120 AD), Pliny (23-79 AD) and Marco Polo (Ca 1250) (Harington 1976). These recordings of asbestos, however, tended to emphasise the novelty value of asbestos woven into "unburnable cloth" and the systematic utilisation of the unusual physical and chemical properties of asbestos were not undertaken at these times. Slowly, however, the potential of asbestos was realised and it is recorded that "everlasting wicks" made of asbestos were manufactured in Norway in the early eighteenth century (Selikoff and Lee 1978). Large scale utilisation of asbestos, however, awaited the industrial revolution when, amongst other things, the invention of the steam engine produced a demand for cheap efficient insulating and packing material; simultaneously a demand was also created for high friction material for the brakes and clutches of this new technology. The enlightened social attitudes of the late 19th Century also brought with them a housing and general construction programme which created an ideal market for asbestos, for example as roofing felt and in cement. To meet this opportunity and capitalise on a new market for a new material, the Canadian chrysotile deposits begun to be worked in earnest in 1876, the first year's output being 50 tons; within 60 years this figure exceeded 1,000 tons per annum.

The same pattern of development was repeated during the late 19th and early 20th Century in other major asbestos mining countries of the world - South Africa, Italy, USSR, USA, Rhodesia and the handful of minor asbestos producing countries (Selikoff and Lee 1978). The description of asbestos as the 20th Century mineral (Gilson 1965) is particularly apt since annual world asbestos output has increased 1,000 fold in the first 60 years of this century being estimated at 4.3×10^6 tons of chrysotile in 1973 (Lincoln 1975) and an annual amphibole production of 2.8×10^5 tons (Hodgson 1975).

This massive asbestos output has been, and is, incorporated into a huge array of manufactured goods and materials. The list is too extensive to attempt here (see Selikoff and Lee 1978) but asbestos products may be broadly grouped into textiles, friction materials, paper products and asbestos cement (Table 1.1). The incorporation of asbestos into such a wide range of products ensured that the maximal benefit accrued from the special properties of asbestos, but it also meant that many different occupational groups and ordinary members of the public were exposed to asbestos and thereby to its associated health hazards. It is notable that risk occurs at both the manufacturing end - miners, spinners, weavers, etc., and user end - construction workers, plumbers, householders.

1.2 Asbestos: shape and surface chemistry

The geology, physics and chemistry of asbestos and its subsequent properties are complex as is mineralogical analysis and a detailed review is beyond the scope of this monograph. The literature on this subject is however extensive and has been brought together periodically as reviews (for example Poncelot et al 1971; Gravatt et al 1978; Michaels and Chissick 1979). In this short review only the major physicochemical features of

TABLE 1.1 Principal industrial uses of asbestos

BROAD GROUPING	EXAMPLE	ASBESTOS PROPERTY UTILISED
Textiles	protective clothing wire/cable insulation packing in pumps pistons filter pads etc.	heat resistance electrical resistance softness/resilience/non-abrasiveness/large surface area/reactivity
Friction material	brake lining clutch facing	heat resistance/non-abrasiveness/resilience
Paper products	roofing felt insulation	heat and rot resistance electrical resistance/ large surface area
Asbestos cement products	roof sheeting pipes	weather and fire resistance/tensile strength resistance to cement alkali/corrosion resistance
		to all of these should be added the property of cheapness

after Lindell 1973

asbestos, considered salient to its biological effects, will be discussed.

Asbestos is the name given to a family of naturally occurring, crystalline, fibrous minerals which differ in chemical composition and are formed by the action of geological forces:- heat, cooling and pressure, in the earth's crust.

Asbestos is divided into two mineralogical types:-

(i) serpentine - comprising chrysotile (white) asbestos, the major asbestos type utilised in industry.

(ii) amphibole - comprising, in decreasing order of commercial importance crocidolite (blue), amosite (brown), anthophyllite, tremolite and actinolite (Hendry 1965; Champness 1976).

1.2.2 Chrysotile ($Mg_6(OH)_8Si_4O_{10}$)

The structure of chrysotile in its natural state is straight silky fibres formed across veins; during industrial processing the fibres are released as white wispy bundles.

At electron microscopy, following even gentle treatment, individual fibres can be seen to be dissociating into the primary fibrils which are hollow. By X-ray diffraction and electron microscopy the tubular structure is not simple but is composed of a rolled up molecular sheet producing a "Swiss roll" appearance in cross section (Hodgson 1965; Zussmann 1978). Chrysotile is typified by a high percentage of Mg, OH and water and the molecular configuration is such that in the rolled sheet structure the $Mg(OH)_2$ (brucite) layer is outermost. The tight packing of the innermost tetrahedral silicon/oxygen layer produces the curvature of the sheet (Gaze 1965). The outer $Mg(OH)_2$ layer provides an active surface for chrysotile which has been implicated in its biological properties (see later).

1.2.2 Crocidolite ($Na_2Fe_2(FeMg)_3Si_8O_{22}(OH)_2$)

Crocidolite is taken as a representative amphibole since it is the one used in this thesis. Crocidolite differs from chrysotile in that its tensile strength is greater, and compared to chrysotile the fibres are straighter, thicker and cannot be defined by single fibre units, unlike chrysotile. The form of crocidolite is probably a consequence of occasional interruptions in the double chain silicate crystal lattice by multiple chains; this structural disturbance may lead to parallel sets of preferential planes of fracture (Whittaker et al 1982).

1.3 Asbestos: Properties

The principal properties of asbestos which led in the first instance to its extensive use in industry were its low thermal conductivity rendering it valuable in insulation, and its relative indestructibility by the common modes of attrition - weather, microbiological, friction etc. These properties are reviewed extensively elsewhere (e.g. Selikoff and Lee 1978) but for the present study only the properties which are considered relevant to the biological activity will be discussed.

1.3.1 Shape

The most obvious property of asbestos is its fibrous shape due, in the case of amphiboles, to systematic disruption of the normal double chain silicate crystal lattice in the native mineral. With chrysotile the strength of individual fibrils is greater than the binding between fibrils and so fracture tends to occur parallel to the fibres. In each case stress then results in partial break-up of the mineral yielding elongated fibrous fragments. This contrasts with the non-fibrous nature of some other dusts which cause lung disease on inhalation such as quartz or coal dust. A fibre is generally defined as a particle with an aspect ratio (length:diameter) of >3. Chrysotile asbestos forms silky fibres

which separate to individual fibrils of 0.02-0.03 μm diameter and variable length. Amphiboles such as crocidolite cleave into coarser, more brittle fibrils down to 0.1 μm diameter (Chowdhury 1975; Selikoff and Lee 1978).

1.3.2 Surface properties

As predicted by physical laws thin fibres have a very high surface:volume ratio and so any effect of mineral fibre on a biological system could be related not only to the fibrous shape but to a factor involving the large surface area which accompanies the fibrous habit.

As discussed above the surface layer of chrysotile is largely $Mg(OH)_2$ which is positively charged at physiological pH, the charge residing in the ionised Mg^+. At lower pH, for example in HCl, the Mg groups are stripped off the fibre surface exposing the silica layer (Morgan et al 1977; Oberdorster et al 1980). In protein containing solution the charge on chrysotile at physiological pH results in adsorption of protein onto the surface (Morgan 1974) with subsequent masking of the surface charge. This adsorption appears to be largely due to the Mg in the brucite layer since the adsorption capacity for protein decreases with HCl (Morgan et al 1977), or EDTA (Valerio et al 1980) mediated leaching in parallel with the surface charge (Light and Wei 1977).

Crocidolite, in keeping with the other amphiboles, has a surface which is essentially silicate and exhibits a negative charge at physiological pH (Light and Wei 1977) but, like chrysotile, trace elements such as Fe, Cr, and Co do leach from crocidolite (Oberdorster et al 1980). In contrast to chrysotile, continued leaching of crocidolite results in an increase in the surface charge (Light and Wei 1977) which presumably increases protein adsorption. While experiments on the leaching of

elements from asbestos are, of necessity, carried out under controlled in vitro conditions it is evident that there is elemental dissolution of asbestos in vivo (Morgan et al 1971; Hart et al 1981; Crawford and Miller 1981).

1.3.3 Aerodynamic properties

With regard to the health hazards of asbestos the most important property asbestos possesses, by virtue of its tendency to break up into small fibres, is its ability to become airborne as a fine dust where it is available for inhalation. This is directly related to the free falling speed of the fibres which is most closely related to diameter; thus the likelihood of any fibre becoming airborne (or settling) is a function of $1/d^2$ where d = diameter of fibre. A fibre of 0.5 μm diameter is thus 16 times more likely to remain airborne than a fibre 2.0 μm in diameter (Acheson and Gardner 1979a). Fibre dimensions also determine which fibre fraction of an inhaled dust will be trapped in the nasal passages, which will be deposited in the alveoli and which will be exhaled without deposition. The behaviour of fibres in this respect is more complex than that of amorphous/spherical particles as shown by the finding that inhaled compact particles 10 μm in diameter never reach the alveoli whereas fibres $>$ 200 μm in length have been found there (Vorwald 1951).

The factors affecting particle deposition in the lung are complex and involve inertial impaction, sedimentation, diffusion, interception and electrostatic precipitation. In pulmonary deposition of asbestos all of these factors apply to varying degrees and have been brought together in a computer generated model by Harris and Fraser (1976); this model shows in general that there is a maximum alveolar deposition

of fibres with equivalent diameter of 0.5-2 μm and of shortest length (25 μm in their model).

1.4 UICC samples

While the basic structure of the asbestos minerals is fairly constant, differences in the proportion of the lesser elements are present in samples of one asbestos type from different regions (Hodgson 1965); also differences in fibre dimensions can be produced by handling or treatment of samples and by different methods of sample collection. The UICC (Union Internationale Contre Cancer) Working Group (1965) therefore recommended that standard asbestos reference samples of respirable size should be prepared from the main asbestos types. It was envisaged that such samples would be extensively characterized and distributed to research centres so that research into asbestos physics, chemistry, biological effects, etc. would be comparable. Such standard reference samples were subsequently prepared (Timbrell et al 1968; Timbrell 1969). The target respirable range which was sought in the UICC samples was <3 μm in diameter and <200 μm in length.

To date these UICC samples have been characterised extensively, for example by gravimetric analysis, atomic absorption spectrometry, neutron activation and emission spectroscopy, optical and electron microscopic size distribution, surface adsorption studies and using many other analyses (Timbrell 1980). In addition the UICC samples have been extensively utilised in numerous experiments into the biological effects of asbestos.

The UICC samples thus introduced a necessary uniformity into the field of asbestos research particularly with regard to biological effects.

1.5 Asbestos related disease

The classical epidemiological approach has contributed greatly to

an understanding of the relationship between asbestos and disease and the following is an introduction to epidemiological evidence for the role of asbestos in disease. This topic has been reviewed extensively elsewhere (Becklake 1976; Selikoff and Lee 1978; Preger 1978).

1.5.1 Asbestosis (Asbestos related pulmonary interstitial fibrosis)

Asbestosis is a chronic lung disease caused by inhalation of asbestos fibres and is characterised by diffuse interstitial fibrosis of the lung. It is a slowly developing and often progressive (continues when exposure ceases) disease characterised by the presence of small irregular pulmonary opacities at X-ray. Accompanying asbestosis, and a consequence of it, can be one or any of the following:- respiratory impairment, dyspnoea, cyanosis, pulmonary rales and finger clubbing. Pulmonary hypertension may be precipitated and right sided heart failure is often the cause of death (DHEW NIOSH 1976).

The first recognition of asbestosis is attributed to Murray in England in 1906 and the first case to appear in the medical literature was reported by Cooke in 1924 (McVittie 1965). By 1930 Merewether and Price had reported to the government that asbestos posed a major problem in the asbestos textile trade and it was realised that in any occupation where workers suffered prolonged exposure to respirable sized asbestos, then a proportion would develop pulmonary fibrosis (Wright 1969).

Since then epidemiological evidence has accumulated linking all asbestos types (Cooper 1978) and asbestos usage in many industries, with the development of asbestosis; for example shipyard workers (Harries 1968); insulation workers (Hammond et al 1979); brake repair and maintenance workers (Lorimer et al 1976) (for a more complete list of occupations at risk see Becklake 1976).

Such a risk necessitated government control of airborne asbestos in

the workplace and in 1933 regulations were introduced rendering death from uncomplicated asbestosis a rarity (Elmes 1981); there are suggestions however that these regulations have not always been stringently enforced (Wright 1969).

Clearly in determining a safe hygiene standard for airborne asbestos in workplaces, information is required on the dose response relationship between exposure to airborne asbestos and development of pulmonary fibrosis. As extensively discussed by Becklake (1976) it is determination of asbestos dose over a lifetime of occupational (and non-occupational) exposure to asbestos which presents the major problem in epidemiological studies into dose response and asbestos disease. However, using several criteria for assessment of dose such as years of exposure, years since first exposure, occupation, occupation and duration and cumulative dust exposure, Becklake cites 14 studies showing a dose response relationship between asbestos exposure and development of lung fibrosis. In a recent well controlled longitudinal study within one factory (Berry and Lewinsohn 1979) the dust levels at various points of the asbestos treatment process were monitored over 20 years and the length of time individual employees worked at these various locations in the factory were taken into account in calculating cumulative dose. The study revealed a clear dose response relationship between cumulative dose and "probable" asbestosis (as judged by X-ray and lung function changes and pulmonary rales).

1.5.2 Carcinoma of the lung

The lung cancer associated with asbestos exposure is bronchogenic carcinoma with the adenocarcinoma cell type predominating with tumours typically located in the lower lobes (Whitwell et al 1974; Preger 1978).

The first identification of a relationship between asbestos exposure and bronchogenic carcinoma is attributed to Gloyne, or Lynch and Smith in

1935 but it was not until Merewether's discovery in 1947 of 13% incidence of bronchogenic cancer in autopsies of employees from the asbestos textile trade that the possibility was seriously considered (Wright 1969). The classical epidemiological work of Doll (1955) confirmed the relationship and indicated a tenfold increase in the expected incidence of bronchogenic cancer in textile workers with at least 20 years of exposure.

Elmes (1981) points out that the 1933 regulations, which led to dust control and the virtual elimination of uncomplicated asbestosis as a primary cause of death, resulted in increased longevity of asbestos workers and the appearance of lung cancer. Thus by 1960 over half of men classified as having asbestosis had lung cancer at autopsy (Buchanan 1965).

Once again Becklake (1976) in her review has comprehensively examined the literature with regard to dose response and cites 16 papers showing a clear dose response relationship between asbestos exposure and lung cancer incidence; dose was once again estimated by the various criteria mentioned above (1.5.1).

With regard to the relative risk of development of lung cancer with exposure to individual fibre types, there is epidemiological evidence that there is a gradation of risk as follows:- amosite> anthophyllite> chrysotile with no data available for crocidolite (Acheson and Gardner 1979a).

Clear evidence for a synergistic association between cigarette smoking and asbestos exposure in development of bronchogenic carcinoma has been demonstrated by extensive epidemiological studies of insulation workers (Hammond et al 1979) and shipyard workers (Elmes 1981).

1.5.3 Mesothelioma

Mesothelioma is a tumour of variable morphology (Davis 1974 a) originating apparently from the mesothelial cells of the pleural lining

of the lungs and chest cavity and from the mesothelial lining of the peritoneal cavity. Mesothelioma is a rare tumour in the general population being estimated at 1 (Becklake 1976) or 2-3 per 10^6 per annum (Preger 1978).

The relationship between exposure to asbestos and development of mesothelioma was established in the classical paper by Wagner et al (1960) showing 33 cases of mesothelioma associated with the Asbestos Hills of the South African Cape crocidolite fields. The relationship was confirmed by Newhouse and Thomson (1965) who also implicated domestic and neighbourhood (of an asbestos factory) exposure. Many epidemiological studies have since confirmed the relationship between mesothelioma and asbestos exposure (for more complete reviews see Acheson and Gardner 1979a; Preger 1979; Kannerstein et al 1978).

Of the 3 major diseases associated with asbestos exposure mesothelioma is the one which has the least clear cut dose response relationship (Becklake 1976; Acheson and Gardner 1979 a) since the associated doses have been claimed to be very small, acquired in non-occupational situations and with very long latent periods. In his model however Peto (1979) finds a dose response relationship between duration of asbestos exposure and risk of death from mesothelioma.

The possibility of fibre transport within the body is suggested by the finding of 27/83 mesotheliomas which originated in the peritoneal cavity in Newhouse and Thomson's (1965) study. A consistent level of peritoneal mesotheliomas have been reported in subsequent epidemiological studies (for example Enticknap and Smither 1964; McEwen et al 1970).

Unlike asbestos associated lung cancer, mesothelioma development is not synergistic with smoking (Whitwell and Radcliffe 1971); the intriguing possibility that chrysotile and the amphiboles could be synergistic in

mesothelioma induction has, however, recently been raised (Acheson and Gardner 1979 b).

1.5.4 Other diseases associated with asbestos exposure

Besides the 3 major lung diseases associated with asbestos exposure, asbestos has been postulated to be involved in several other diseases.

Gastrointestinal carcinoma Epidemiological evidence has accumulated implicating asbestos exposure with increased levels of gastrointestinal tract carcinoma (for review see Schneidermann 1974) and this now represents the second greatest mortality risk in asbestos workers after lung cancer (Becklake 1976). The site of this excess gastrointestinal cancer include the oesophagus, stomach and colon/rectum (Selikoff et al 1973). The gut, as a site for asbestos carcinogenesis, may reflect the gastrointestinal ingestion of asbestos inherent in the normal pulmonary clearance mechanism.

Laryngeal carcinoma Several studies have suggested an association between cancer of the larynx and asbestos (for example Stell and McGill 1973).

Haemopoetic system neoplasia Gerber (1970) and Kagan (1980) have reported cancer of the haemopoetic system in asbestos exposed individuals but Selikoff and Lee (1978) point out that this is not in accord with their extensive experience in asbestos related mortality at Mount Sinai, New York.

Pleural change This is a category which includes benign, possibly premalignant, thickened, hyaline and calcified pleural plaques. This group of associated lesions, present in the parietal pleura, are found in association with asbestos exposure (Preger 1978). The lesions are a result of local connective tissue proliferation and the mesothelial

covering may also undergo metaplasia (Lewinsohn 1974) suggesting that this may be the origin of sites of mesothelioma. Benign pleural effusion is often an accompanying phenomenon to these types of pleural change (Becklake 1976).

Asbestos bodies Asbestos bodies are not a disease state but they represent a phenomenon which does not fit easily into any categorisation of the biological effects of asbestos.

Asbestos bodies are fibres which have attained a conspicuous protein coating by residence usually in lung tissue although they are found in other sites (Selikoff and Lee 1978). Asbestos bodies appear to be formed by the laying down of mucopolysaccharide followed by ferritin, around fibres ingested by macrophages with final release of the asbestos body (Davis 1965, 1970).

Such bodies are frequently found in the lungs of asbestos exposed persons (Ashcroft and Heppleston 1973) and city dwellers (Oldham 1973). The exact relationship between asbestos body concentration in the lungs, and fibrosis, is not a simple dose response one (Ashcroft and Heppleston 1973) which is not surprising since the ratio of coated to uncoated fibres may be as low as 1:11,000 (Davis and Gross 1973). Asbestos bodies seem therefore to represent a partial defence reaction of the body to inactivate foreign mineral fibres; this may operate successfully for a low number of inhaled fibres but the mechanism cannot apparently cope with greater challenge and lung damage ensues.

1.6 Asbestos related disturbance to the immune system

Disturbance of the normal immune profile is not a disease state but the central role of the immune system in disease processes demands consideration of immune parameters to fully understand the aetiology

and development of any disease. Epidemiological evidence for the role of altered immune status in the development of asbestos related disease was scanty up to 1973 when the Report of the Advisory Committee on Asbestos Cancer to the Director of the International Agency for Research on Cancer (Report 1973) recommended enquiring into the immunocompetence of asbestotics with regard to the role of immunity in the subsequent development of neoplasms. Since then there have been several epidemiological studies indicating definite alterations in the immune profiles of asbestos workers and patients suffering from asbestos related disease and these will be reviewed.

1.6.1 Cell mediated immunity

In the simplest approach the number of circulating white cells have been counted and relative leukopaenia has been reported in asbestos workers (El Sewefy et al 1974) and asbestos miners and quarriers (Munan et al 1981).

Further refinement, involving enumeration of circulating T lymphocytes by the E rosetting technique, has suggested that the total T lymphocyte count is reduced in asbestotics (Kang et al 1974; Kagan et al 1977b)although reports of unchanged levels of T cells in asbestotics (Gaumer et al 1981) and increased T cells in asbestotics who smoked (Wagner et al 1979) have also been reported.

No reports have been published involving use of the newly available panels of monoclonal antibodies to T cell subsets to further dissect the T cell status of asbestos exposed individuals but the use of the "late rosetting" T cell technique has been used as a marker for T suppressor cells. In two studies, using this technique, evidence has been obtained for decreased numbers of suppressor T cells in asbestotics (Wagner et al 1979; Gaumer et al 1981).

To obtain information on the functional state of the peripheral blood T cells the mitogenic response to Phytohaemagglutinin (PHA) or Concanavalin A (Con A) has been utilised in several studies. Using PHA, uptake of thymidine by T lymphocytes has been found to be decreased in asbestotics (Kang et al 1974; Kagan et al 1977a; Haslam et al 1978; Wagner et al 1979). In contrast Gaumer et al (1981) found no difference in Con A response in 10 asbestotics compared to 10 suitable controls while Lange's (1980) data on PHA transformation is impossible to interpret due to variability in the response of asbestotics. Of particular interest was the finding by Kagan et al (1977a)of an inhibitor of PHA transformation in the serum of some asbestotics.

In their extensive study Kagan et al (1977a)measured "lymphocyte mediated cytotoxicity" and found it also to be significantly decreased in asbestotics.

As a measure of total cell mediated immunity the ability of asbestos workers to mount a delayed hypersensitivity response to recall antigens has been measured in some studies. In all the studies where this has been done significantly increased anergy to recall antigens has been demonstrated (Kagan et al 1977a; Lange et al 1978; Gaumer et al 1981). In a further refinement Lange (1980) measured the generation of migration inhibition factor (MIF) following challenge with recall antigens and found a significant reduction in MIF production by asbestotics compared to controls.

1.6.2 Humoral immunity

Gaumer et al (1981) have described an increase in the number of B lymphocytes in asbestotics compared to controls while Kagan et al (1977 b) found no difference in this parameter. There is, however,

evidence from several studies, of a stimulation in humoral immunity manifest as significantly increased levels of serum IgA, IgG, IgM and IgE in asbestotics (Kagan et al 1977 b; Lange 1980; Huuskonen et al 1978) although not all of these studies found increases in all of the Ig classes in their experimental groups.

Kagan et al (1977b) additionally reported increased secretory (salivary) IgA and El Sewefy and Hassan (1971) reported immunoelectrophoretic evidence of increased IgG, IgM and IgA, in asbestotics.

Possibly related to the above reports of elevated circulating Ig in asbestotics is recurrent evidence of autoimmune disease manifest as serum anti-nuclear antibody and/or rheumatoid factor (Pernis et al 1965; Turner-Warwick and Parkes 1970; Kagan et al 1977 b; Lange et al 1978). There is no organ specificity in these auto-antibodies as typified by the failure to find any evidence of specific anti-lung activity in the asbestotics studied by Turner-Warwick and Haslam (1971).

These findings of autoimmunity in asbestotics taken together with the results of a study by Toivanen et al (1976) who found no evidence for increased anti-nuclear antibody or rheumatoid factor in 66 asbestos workers without asbestosis, and Edge (1976) who failed to demonstrate increases in these parameters in 160 asbestos workers with pleural plaques and no asbestosis, supports the view that autoimmunity is a result of asbestosis rather than a contributory cause. Militating against this view however is the work of Huuskonen (1978) who found increased auto-antibodies in asbestos workers compared to controls but not in asbestotic, compared to non-asbestotic, asbestos workers. One possible explanation of the conflicting results of these epidemiological studies is the method of assessing sub-clinical asbestosis amongst workers in view of the difficulty in interpretation of X-ray data.

Additional evidence of inflammation and immunostimulation in asbestotics is present in the work of Huuskonen (1980) who reported increased serum C_3 and C_4 and $\alpha 1$ anti-trypsin in asbestotics compared to non-asbestotic asbestos workers.

The HLA tissue type of individuals has been found to play a role in susceptibility to some diseases and Merchant et al (1975) reported increased incidence of HLA W27 antigen in asbestotics suggesting that HLA W27 individuals had increased susceptibility. In the same study Merchant et al failed to confirm the suggestion by Badr and El Sewefy (1971) that asbestosis was associated with Blood Group 'O'. Evans et al (1977) failed to repeat the findings of Merchant et al with regard to HLA W27 in a group of asbestotics and controls and instead found increased fibrosis to be linked with HLA B12; they also found that HLA BW5 was much reduced in asbestotics and interpreted this as HLA BW5 conferring "protection" against asbestosis.

Studies on the immunocompetence of asbestos exposed persons who developed mesothelioma have revealed a decrease in the number of circulating T cells but no evidence of impaired cell mediated immunity as measured by antigen recall, or T cell mitogenesis (Ramachandar et al 1975; Haslam et al 1978).

Epidemiological studies into immune status in asbestos related disease can be summarised as follows. The presence of asbestosis is accompanied by impaired T cell function and cellular immunity. Selective loss of T suppressor cells reported by two groups may have a role in stimulation of humoral immunity present along with asbestosis and also possibly in the manifestation of autoimmunity consistently detected in asbestotics. It is not clear whether asbestosis predisposes to auto-immune disease or vice versa and further studies are required to clarify the conflicting evidence on this point. The reports of lack of

impairment in T lymphoproliferative status in mesothelioma patients is in contrast to the findings of decreases in this function reported in many studies of cancer patients (Herberman 1978). As such, this particular finding may provide some insight into the role of the immune response in development of this unusual tumour.

1.7 Portals of entry

Once epidemiological evidence had demonstrated that asbestos exposure was associated with defined diseases the subject was accessible to application of experimental pathology using suitable models. It is apparent that the lungs represent the major portal of entry and target organ for the pathological effects of asbestos in view of the ability of asbestos to become airborne and be inhaled. It is not however the only target for asbestos disease since asbestos inhaled is asbestos ingested via the gastrointestinal tract due to the fact that most of the dust which settles before the terminal airspaces, and much of that which does, ends up being cleared from the lung via the mucociliary escalator. This system comprises a continuous blanket of mucus (plus particles) which is constantly being propelled upwards towards the throat, through the action of ciliated epithelial lining cells, where it is normally swallowed.

Additionally asbestos can be ingested directly into the gastro-intestinal tract as a contaminant of drinking water (Cunningham and Pontefract 1971), water filtered through asbestos based filters (Selikoff and Lee 1978) or in beer, sherry, vermouth or soft drinks (Cunningham and Pontefract 1971). Asbestos can also be injected as a contaminant of therapeutic preparations (Selikoff and Lee 1978).

These other portals of entry could be contributory factors in the extra-pulmonary neoplasia associated with asbestos but the lung remains

the site of the 3 major asbestos related diseases, pulmonary asbestosis, bronchial carcinoma and pleural mesothelioma.

1.8 Experimental pathology

Confirmation of the association between an agent and a disease, which is suspected from epidemiological evidence, requires the testing of the suspected agent in experimental animal models on the assumption that similar effects will be observed. In addition the use of animal models allows manipulation of the suspected agent, and the laboratory animal which can aid in dissecting out the elements of the disease process. In the following section the evidence gained from experimental pathology as to the role of asbestos in disease will be reviewed.

1.8.1 Fibrosis

Experiments to determine the fibrogenic effects of asbestos in animals were begun almost as soon as asbestos was suspected as a pathogenic agent through the report of Cooke in 1924. Thus Wagner (1963) records that Mavrogordato, in 1925, dusted guinea pigs with chrysotile for 2 hours a day for 50 days and that one animal died with pulmonary fibrosis and asbestos bodies. Wagner (1963) also reviews the extended study of Gardner and Cummings which began in 1928 and which reported peri-bronchiolar fibrosis in guinea pigs dusted with chrysotile for 600 days. In these experiments only chrysotile was used but by 1951 Vorwald et al had reported instillation of crocidolite, chrysotile and amosite into the lungs of rats and similar lesions were produced by all asbestos types. Holt et al (1965) found similar levels of fibrosis and type of lesion following inhalation of crocidolite, chrysotile, amosite and anthophyllite in guinea pigs. It was not until 1974 however that an attempt was made using UICC samples to accurately assess the two major parameters, i.e. level

of dust exposure and degree of fibrosis. Thus Wagner et al (1974) maintained their dust level at 10 mg/m^3 and assessed the degree of fibrosis blind on a scale from 1-8; these workers reported that chrysotile was significantly more fibrogenic in rats than amosite and that crocidolite was intermediate. Asbestosis was found to be progressive after cessation of dusting. In the study of Davis et al (1978) UICC samples were applied in clouds of equal fibre number or equal mass and in this study, mass for mass, chrysotile was found to be twice as fibrogenic as the amphiboles.

Once the essential fibrogenic activity of asbestos was confirmed in animals the properties of fibres which imbued them with their fibrogenic potential was investigated. Thus King et al (1946) showed that short fibre chrysotile (< 2.5 µm) produced a lesser reaction than a long fibre sample (> 15 µm) and Klosterkotter (1968) reported that both crocidolite and chrysotile lost their fibrogenic potential when ground to less than 5 µm. Timbrell and Skidmore (1968) obtained a greater pulmonary reaction to inhaled long fibre amosite than to a short fibre amosite produced by "further grinding" of the long sample; the clouds were of equal mass. Davis (1972a) reported that a variety of dusts including chrysotile, injected into the murine peritoneal cavity, were more fibrogenic as long fibre samples than as samples ultrasonicated or ground and sieved to remove the long fibre fraction. Wright and Küschner (1977) instilled into the lungs of rats, well characterised long and short fibre samples of crocidolite and found extensive fibrosis with the long fibres and no fibrosis with the short fibre sample.

While long fibres seem to be the most active in fibrogenesis by asbestos there are isolated reports of short fibre samples causing extensive fibrosis (for example Holt et al 1965).

A few studies have investigated the role of the asbestos fibre surface in fibrogenesis; polyvinyl-pyridine-n-oxide, which produces protection against silica fibrosis by masking the surface OH groups, was found to have no effect on the in vivo fibrogenicity of asbestos (Davis 1972b). Heating chrysotile to above 400°C and then injecting it into the peritoneal cavity resulted in a decrease in fibrogenicity as measured by size of fibrous granulomas (Davis and Coniam 1973); the authors concluded that the loss of the fibrous structure may have been as important as direct effects of heat on the chrysotile surface.

1.8.2 Carcinogenicity - asbestos alone

Following his pioneering study revealing increased incidence of mesothelioma in the Cape asbestos fields, Wagner (1962) injected asbestos intrapleurally into rats and produced pleural mesotheliomas. Wagner and Berry (1969) went on to report mesothelioma induction by crocidolite, chrysotile, amosite, anthophyllite and brucite administered in the same way. Once again, having confirmed that animal experimentation mimmicked the human situation, in terms of mesothelial reactivity, experiments were carried out aimed at elucidating the main parameters of the fibre involved in carcinogenesis.

Stanton and Wrench (1972) carried out a study on the role of fibre geometry on mesothelioma production by fibrous dusts. Using an unusual system of applying fibre directly to the pleural surface in a gelatin base on glass fibre pledgets, a method that appears to only have been used by Stanton's group, these workers found that crocidolite, chrysotile and amosite all produced mesotheliomas and that milling to shorter length reduced the tumourigenic potency of crocidolite. In subsequent studies Stanton and co-workers (1977, 1978) went on to show that, in

their system, fibres <0.25 μm diameter and >8 μm in length were the most potentially tumourigenic. Wagner et al (1970) confirmed the increased potency of long thin fibres, compared to short thick fibres, in their intrapleural injection studies.

Inhalation of asbestos has been used to generate tumours in experimental animals but since even in occupationally exposed human populations the incidence of lung tumours is low (particularly when the synergistic effect of smoking is absent), this is an inefficient way of producing tumours, albeit the most realistic way.

Gross et al (1967) produced lung carcinomas and a single mesothelioma in rats inhaling chrysotile while Reeves et al (1974) reported the same neoplasms in rats exposed to clouds of chrysotile, crocidolite and amosite. Wagner et al (1974) exposed rats to clouds of UICC asbestos of all types and produced lung carcinomas and mesotheliomas. Davis et al (1978) reported a study where rats were exposed to equal masses and equal fibre numbers of UICC crocidolite, chrysotile and amosite; compared on this basis chrysotile was the most carcinogenic producing lung carcinomas and mesotheliomas. In this study crocidolite produced no carcinomas and one mesothelioma.

Several studies have been carried out in an attempt to dissect out the major factor(s) in fibre responsible for the carcinogenicity. Morgan et al (1977) showed that extensive acid leaching of chrysotile reduced its tumourigenic potential by approximately 25% and, while the authors claim that this treatment did not affect the tendency of the chrysotile to fragment, there is evidence that such treatment increases the fragility of chrysotile (Selikoff and Lee 1978). Monchaux et al (1981) have also reported virtual abolition of tumourigenic potency of chrysotile with acid leaching. Early suggestions that polycyclic

hydrocarbons contaminating fibres could be involved in asbestos carcinogenesis (Harington 1965) were ruled out when vigorous extraction of contaminating and natural oils failed to alter asbestos carcinogenicity (Wagner et al (1970). The possibility that naturally occurring or contaminating carcinogenic metals such as nickel and cobalt could be a factor in carcinogenesis was studied by Stanton's group; nickel, chromium and other carcinogenic metals were attached to non-fibrous particles of appropriate size and this failed to render the particles carcinogenic (Stanton and Layard 1978).

Immunobiology of mesothelioma

In recent years the Wagners and others have carried out studies into the immunobiology of mesothelioma in rats. Berry and Wagner (1976) reported that age at asbestos inoculation had a significant effect on rate of mesothelioma induction; rats injected at 10 months of age had a significantly increased mesothelioma incidence compared to those injected at 2 months. The age related difference in susceptibility was reported by the authors to be in keeping with similar studies using other experimental carcinogens. Wagner (1979) thymectomized and sham-thymectomized rats and measured crocidolite induced mesothelioma production. This study revealed that sham-thymectomy was the major effect in reducing mesothelioma rate and that sham-thymectomy was significantly more effective than thymectomy in reducing mesothelioma production. Wagner advanced several explanations for these results, the most likely being that surgery caused T cell dependent macrophage activation to cytotoxicity which was abrogated by thymectomy. Wagner et al (1980) also reported that BCG, silica and talc given by intrapleural injection following intrapleural crocidolite had no effect on

mesothelioma incidence while similarly applied carageenan caused a threefold increase in mesothelioma incidence. The authors point out that carageenan kills macrophages and that such toxicity, or altered macrophage function, could have contributed to mesothelioma development. In a recent study carageenan, injected intraperitoneally, has been shown to potentiate growth of a concomitant experimental peritoneal ascitic lymphoma (Pugh-Humphreys et al 1977).

Brown et al (1980) studied rat mesothelioma tumour lines, maintained through 20 transplant generations by subcutaneous trochar implantation. Rats immunised by excision of subcutaneous tumours, or implanted with irradiated tumour fragments, plus suitable controls, were challenged with tumour fragments; no protection was conferred by these treatments thus failing to demonstrate tumour associated transplant antigens in asbestos induced mesothelioma. Using a different index of immunogenicity for rat mesotheliomas, Stevens et al (1981) failed to detect antibody dependent cell mediated cytotoxicity mediating activity in serum from mesothelioma bearing rats using bowel adenocarcinoma cells as targets and normal peripheral blood lymphocytes as effectors.

In the same assay system serum from chemically induced tumour bearing rats was positive (approximately 14% cytotoxicity). These studies demonstrate that experimental asbestos mesotheliomas do not elicit cell mediated immune responses as manifest by tumour rejection following immunisation and challenge, or humoral immune response which can mediate antibody dependent cell mediated cytotoxicity.

In a study into the uses of carcinoembryonic antigen (CEA) phenotype in tumour diagnosis Whitaker and Shilkin (1981) reported absence of CEA in 40 human mesotheliomas and its presence in carcinomas; while of value in diagnosing suspect tumours this finding also underlines the mesenchymal origin of mesotheliomas since CEA is a marker of epithelial neoplasms

(Goldenberg et al 1978). Gormley et al (1980b) provided further evidence for the mesenchymal origin of experimental asbestos mesotheliomas when cell lines from mesotheliomas of variable histological type tended to adopt a fibroblastic appearance on culture suggesting a common mesenchymal heritage.

1.8.3 Carcinogenicity - co-carcinogenic effects

The synergistic, or co-carcinogenic interaction of asbestos and cigarette smoking in the genesis of bronchial carcinoma which was demonstrated by epidemiological methods prompted Wehner et al (1975) to expose hamsters to (i) cigarette smoke, (ii) chrysotile or (iii) chrysotile plus cigarette smoke. Due to excessive fibrosis in the asbestos exposed group, however, dusting had to be discontinued early and, possibly due to conflicting mortality from asbestosis, the group receiving smoke and asbestos had significantly fewer lung tumours than the "cigarette smoke only" group. In another poorly controlled study (Humphrey et al 1981) 9 dogs received intratracheal instillations of crocidolite for up to 3 years; 7/9 of these dogs smoked 45 cigarettes per week for 6 years. Since the two dogs which received asbestos alone produced mesotheliomas this rather confounded the exercise as a study in synergism. However, 4/7 of dogs receiving asbestos only and 3/7 of dogs receiving asbestos and smoke, developed bronchial carcinomas.

Several studies have investigated co-carcinogenic interaction of asbestos with conventional laboratory carcinogens. Pylev and Shabad (1973) produced 0% tumours in rats receiving intratracheal chrysotile (2mg) and 0% tumours in rats receiving 5 mg Benzo (α) pyrene, the major hydrocarbon in cigarette smoke. Rats receiving 2 mg chrysotile with Benzo (α) pyrene adsorbed onto the surface produced 29% with tumours, while rats receiving 2 mg chrysotile plus 5 mg benzo (α) pyrene

developed 59% tumours. Using tracheal transplants exposed to various non-tumourigenic doses of dimethylbenzanthracene in pellet form, followed by a non-tumourigenic dose of chrysotile in a gelatin pellet, Topping and Nettesheim (1980) demonstrated up to 23% incidence of tracheal carcinomas. These authors present their results as evidence for a promoting effect of asbestos as the second step to initiation by dimethylbenzanthracene and point out that the temporal sequence of application used in these experiments precludes a "carrier effect" of asbestos in targetting the dimethylbenzanthracene to susceptible cells.

In an attempt to demonstrate a synergistic role for asbestos in gastrointestinal carcinogenesis Ward et al (1980) utilised the rat model whereby subcutaneous azoxymethane produces intestinal tumours in rats. Intra-gastric instillation of UICC chrysotile was given concurrently with subcutaneous azoxymethane, but no significant excess of tumour was found in the group over and above the level found in the group receiving azoxymethane alone.

Kanazawa et al (1979), prompted by the report of Chabot et al (1970) on viral induction of mesothelioma in fowl, undertook a series of experiments to ascertain whether asbestos could potentiate viral oncogenesis. Mice given 5 µg UICC chrysotile produced no tumours (0/60); 1/59 of mice receiving Moloney sarcoma virus only produced a single tumour (which regressed); 44/61 of mice receiving 5 µg chrysotile plus virus (at the same dose as the controls) produced tumours and 22 died within 100 days. It was notable that quartz and carbon administered along with virus produced a low level of tumours:- quartz 12/62 - 2 lethal; carbon 8/62 - 1 lethal.

Experiments into asbestos co-carcinogenesis with polycyclic aromatic hydrocarbons, which do not fit easily into experimental pathology or cell studies, include those using liver cell microsomal fractions to

study carcinogen metabolism. Thomson et al (1978) found that ferric ions (a major component of asbestos) at low concentrations caused a 20% increase in the binding of benzopyrene to DNA and made the rather tenuous association that ferric ions leaching from asbestos could be synergistic to benzopyrene carcinogenesis. Although Thomson et al did not actually test asbestos in their system, Kandaswami and O'Brien (1980) reported that benzopyrene adsorbed onto asbestos was dispersed in microsomal lipid fraction to a greater degree than benzopyrene alone; this finding lent support to a similar observation by Lakowicz and Mylden (1978) using artificial membranes. Kandaswami and O'Brien also reported that all 4 main types of asbestos, with benzopyrene adsorbed, inhibited aryl hydrocarbon hydroxylase in rat liver microsomal fraction. Since this enzyme is responsible for metabolism of benzopyrene, then its suppression could enhance the retention time of adsorbed benzopyrene so increasing the carcinogenic potential. Since Naseem et al (1978) have reported increased inducibility of aryl hydrocarbon hydroxylase in the lymphocytes of asbestos workers, and implicated this in asbestos carcinogenesis, it appears that both induction and suppression of this enzyme is being blamed for asbestos carcinogenesis.

Asbestos has therefore been shown experimentally to act as a co-carcinogen, probably a promoter, with some common polycyclic hydrocarbons as initiators. There is evidence that the asbestos could act at several levels:- (i) as a carrier for the polycyclic hydrocarbons; (ii) as an aid to dispersion of hydrocarbon in the lipid phase; (iii) through modulating the expression of aryl hydrocarbon hydroxylase. Asbestos apparently can also act synergistically with oncogenic virus in producing tumours. These findings have important ramifications for occupational hygiene and experimental asbestos carcinogenesis. Thus, although contaminating hydrocarbons appear not to be involved in UICC asbestos carcinogenesis (Wagner and Berry 1969) the possibility of contamination of

asbestos in occupational situations, or concomitant exposure, must be a possibility. The presence of virus infection in men and animals is also an unknown quantity and the possibility that asbestos affects oncogenic virus expression remains a possibility.

1.9 Cytotoxicity

The ability of asbestos to kill cells was the major *in vitro* effect of asbestos which was reported up to the middle seventies. The exact role of asbestos cytotoxicity *in vitro* to the development of asbestos associated disease remained obscure but a large body of research was carried out measuring the different parameters associated with the ability of asbestos to kill and disrupt cells.

1.9.1 Haemolysis

McNab and Harington (1967) described the haemolytic effect of chrysotile asbestos on erythrocytes and the rather lesser haemolytic activity of the amphiboles (Harington *et al* 1971). It was suggested that the haemolytic activity of chrysotile was due to its positively charged, magnesium rich surface which interacts with the negatively charged sialic acid residues of the erythrocyte membrane causing glycoprotein clustering and osmotic disruption (Harington *et al* 1975). This theory of the haemolytic action of chrysotile has recently gained support from the work of Sykes *et al* (1980). Asbestos haemolysis occurs only in serum free conditions since fibres rapidly become protein coated, and the active surface masked in protein solution. Abolition of haemolysis by serum (Allison 1973), cell fragments (Ottery and Gormley 1978) and lipids (Jaurand *et al* 1979) makes it unlikely that haemolysis has any direct relevance to the *in vivo* situation. The directly disruptive action of asbestos on membrane has, however, been

allocated a role in macrophage cytotoxicity by chrysotile.

1.9.2 Cytotoxicity to macrophages

Harington et al (1974) reviewed studies on the cytotoxic effects of asbestos on macrophages in culture between 1968 and 1973. They showed that in 11 studies using rat, mouse, guinea pig and hamster macrophages exposed to doses of asbestos from 20-1,000 $\mu g/10^6$ cells, remarkably consistent results were obtained. Thus chrysotile was shown to have marked rapid toxicity in serum free conditions and delayed cytotoxicity in the presence of serum. In 5/6 of the studies where the comparison was made, chrysotile was more cytotoxic than amphiboles. According to Allison (1971, 1973) and Harington (1976) the early toxicity in serum free conditions is analogous to haemolysis and the late cytotoxicity in serum conditions is due to the membranolytic effect of intralysosomal chrysotile on the lysosomal membrane causing leakage of lytic enzymes into the cytoplasm and cell death. This theory of asbestos toxicity was based on Allison's theory of silica toxicity to macrophages and his statement of 1971 sums up the importance then placed on asbestos cytotoxicity and the rather vague association between alveolar macrophage toxicity and fibrosis:-

> "The slowly progressive damage to macrophages so produced may be an important factor in the fibrotic reaction to asbestos."
>
> (Allison 1971)

Harington could also write in 1974 with apparent confidence that -

> "the death of the macrophage is a pre-requisite to the subsequent fibrogenesis although the biochemical steps between the two major processes have not yet been worked out."
>
> (Harington 1974)

It is notable that Allison's lysosomal rupture theory of silica induced cell death has been comprehensively tested recently (Kane et al 1980) and shown to be deficient in its major premise and that lysosomal

rupture can be dissociated from cell death. These authors suggest that the target for silica toxicity, even in the presence of serum, is the cell membrane and that lysosomal rupture is not the major factor in silica induced macrophage killing. This suggests that the mechanism of chrysotile cytotoxicity to macrophages *in vitro* must be similarly re-examined.

In 1974 Davies *et al* showed that macrophages could be stimulated by asbestos to release lysosomal enzymes in the absence of cell death and this effect has been confirmed (Morgan *et al* 1978; Morgan and Allison 1980; Jaurand *et al* 1980). Since the central role of stimulated or activated macrophages in chronic inflammation and immunological function had been fully recognised during the early seventies, followed by the recognition that normal living macrophages could release fibroblast stimulating factors, there has been a shift away from macrophage death to stimulatory effects of asbestos. This tendency to emphasise the stimulatory effects of asbestos on macrophages has been led by Miller and co-workers (1976, 1977, 1980) who, in contrast to Allison, emphasised the differences between silica and asbestos (Miller 1979) and the resulting differences in macrophage response in immunity; and by Hamilton and co-workers (1976, 1980, 1981) who highlighted the potential role of asbestos activated macrophages in chronic inflammation.

Studies on the cytotoxic effects of asbestos have continued, however, but with a tendency to use permanent macrophage-like cell lines (see papers in Brown *et al* Eds. 1980) and using cytotoxicity as a potential predictor of fibrogenicity in the screening of potentially hazardous dusts. The dangers of such a predictive test, if used alone, were succinctly expressed by Harington *et al* (1975):-

> "all cytotoxic types (of asbestos) are fibrogenic but not all fibrogenic types are cytotoxic"

the dangers of correlating cytotoxicity with fibrogenicity have been more recently emphasised by Le Bouffant et al (1980).

1.9.3 Asbestos toxicity to other cell types

Asbestos has been reported to be cytotoxic to several other cell types such as fibroblasts (Allison 1973; Chamberlain and Brown 1978), mesothelial cells (Allison 1973; Kaplan et al 1980a) and epithelial cell lines (Neugut et al 1978; Craighead et al 1980).

1.9.4 Cytotoxicity and carcinogenicity testing

The simplicity and cheapness of an in vitro system for detecting potential carcinogenic activity of mineral dusts has resulted in research into the feasibility of using cytotoxicity, to suitable targets, as a possible correlate of tumourigenic potential. Chamberlain and Brown (1978) used a panel of dusts against a hamster lung epithelial cell line and an alveolar type II lung epithelial cell line. These workers reported that quartz, a non-carcinogenic but highly cytotoxic dust to macrophages, was not cytotoxic but that UICC asbestos samples were very cytotoxic. This work was extended (Brown et al 1978) to show that the best correlate of cytotoxicity in 3 UICC amphiboles was the number of fibres longer than 6.3 μm. This figure comes quite close to the length cited by Stanton and his co-workers to be most carcinogenic in animal studies, i.e. longer than 8 μm. On this basis these workers suggested that, in their cell system, activity correlated with the tumourigenicity of fibrous minerals rather than their fibrogenic activity. Similar claims have been made for the macrophage-like cell line P388D1 with regard to cytotoxic discrimination between Dawsonite samples of varying aspect ratio (Wade et al 1980). It should be noted, however, that another study (Gormley et al (1980a) urged caution in correlating cytotoxicity in P388D1 cells with tumourigenic potential of some asbestos types.

It seems reasonable that, as suggested in the concluding remarks to a recent conference (Brown et al Eds. 1980) that before making any claims that a cell line can discriminate, on the grounds of its cytotoxic response, between tumourigenic dusts, it should be tested, in several centres, against a panel of non-pathogenic, fibrogenic, non-carcinogenic and carcinogenic dusts.

1.9.5 Survival versus cytotoxicity in asbestos induced fibrosis and carcinogenesis

In in vitro studies into the effects of asbestos on defined cell populations, much emphasis has been placed on the ability of asbestos to kill cells. It seems logical, however, that the surviving cells are an important population in disorders of cell growth control such as fibrosis and cancer.

With regard to fibrosis a role for macrophage cytotoxicity is often implied but the weight of evidence is that asbestos, even chrysotile, is not toxic to macrophages in vivo (Miller 1978) while in vitro the amphiboles are only weakly cytotoxic and chrysotile cytotoxicity may be a transient phenomenon decreasing with magnesium leaching. It is also possible that over dose effects, compared to the in vivo situation, can be misleading in in vitro experiments. It is notable that many of the "harmful" mediators traditionally seen as being released from dead or dying macrophages - elastase, collagenase, lysosomal hydrolases, oxygen radicals, fibrogenic factors, etc. (Brain 1980) are in fact released in increased amounts by activated macrophages (Davies and Bonney 1980; Pabst and Johnson 1980; Wahl et al 1980a).

In asbestos carcinogenesis, direct cytotoxicity to relevant target cells (epithelial cells, mesothelial cells) may be important in inducing

repair and proliferation which could lead to transformation (Kakunaga 1975). It has however been suggested that cytotoxic agents can create selective pressure in favour of "initiated" cells (Solt and Farber 1976) and so the cells which survive asbestos treatment, but are changed such that they go on to form tumours, are obvious targets for study. Similarly macrophages surviving asbestos challenge (or simply present in dusted animals) in view of the cell growth regulatory role of the macrophage (Keller 1981) deserve attention as to any possible alterations in this function which could play a role in asbestos carcinogenesis and fibrosis.

Thus, while some cells in the lung may die following asbestos deposition, and in the peritoneal cavity following asbestos injection, it is a central argument of this thesis that the living cells can be considered to be as important as the dead ones in bringing about subsequent disease.

1.10 Potential targets for asbestos in the lung

Given that the major locus for the pathological effects of asbestos is the lung, it is possible to list the cell populations and systems in the lung which, on encountering asbestos, could be altered in such a way as to result in disease. This then allows studies to be carried out on relevant isolated systems or homogeneous cell populations to further dissect out the effects of asbestos. These effects could be directly on fibroblasts to produce fibrosis, directly on epithelium to produce carcinoma, or directly on mesothelium to produce mesothelioma. Asbestos could also act indirectly via the cells and humoral elements of the major body defence systems, the immune and inflammatory systems, to bring about overall changes in the growth control of the above mentioned target cells.

In the following sections it is intended to (i) review the evidence for direct effects of asbestos on these target cells with regard to changes which could be relevant to fibrosis or carcinogenesis; (ii) review the role of the macrophage in inflammation and immunity with emphasis on the known effects of asbestos on macrophage function; (iii) review the tumour cell modulating effects of macrophages.

1.10.1 Direct effects - fibroblasts

Using rabbit lung fibroblasts in vitro Richards et al (1971) and Richards and Morris (1973) have shown that, following an initial cytotoxic effect, 3 weeks of culture with chrysotile results in increased collagen secretion by these cells. This group also grew fibroblasts for 8-9 months in the presence of chrysotile and this gave rise to a strain of fibroblasts which produced greater amounts of collagen than control fibroblasts maintained in parallel for the same length of time (Hext et al 1977). The early cytotoxic effect of chrysotile in this system was examined closely (Richards and Jacoby 1976) and the recovery of the cultures was seen to be typified by gross changes in the pattern of collagen deposition from an even distribution in controls, to short thick clusters in the long term chrysotile treated cultures. The authors suggest that such altered collagen may contribute to fibrosis by being more stable to normal turnover than conventional collagen. Richards et al (1977) went on to postulate a mechanism for chrysotile induced fibroblast stimulation involving entry of chrysotile into fibroblasts in both membrane bound (lysosomal) and non-membrane bound forms. Adsorption of RNA by the free chrysotile is envisaged as depleting the cytoplasmic RNA pool and causing stimulation to produce more RNA and hence increased protein synthesis; this is coupled with removal of lysosomal degradative enzymes by adsorption to lysosomal

chrysotile so inhibiting the normal collagen degradative process. While rather mechanistic this model could operate and is open to experimental verification; it is however tempting to speculate that the many fibroblast membrane receptors which have been recently described (Pastan and Willingham 1981) are targets for direct effects of asbestos on fibroblasts.

In a different approach to the direct effects of asbestos on fibroblasts Maroudas et al (1973) utilised the fact that normal fibroblasts require a solid support in order to undergo proliferation, i.e. they are anchorage dependent; these authors then examined the threshold length of asbestos which would provide sufficient anchorage for fibroblast proliferation in semi-solid agar. The authors found that only asbestos samples with fibres more than 20 μm long supplied sufficient anchorage for fibroblast proliferation. While these authors compared these results with their findings on the increased ability of longer fibres to cause mesotheliomas, it seems as likely that such a mechanism could operate in fibrosis.

Several studies have shown chromosomal alteration following treatment of fibroblastic cells (Chinese hamster ovary cells) with asbestos in vitro; thus chromosomal structural changes and aberrations were found with low doses (10 μg/ml) of both chrysotile and crocidolite (Lavappa et al 1975; Sincock and Seabright 1975) while only crocidolite (10 μg/ml) was found to cause significant increases in sister chromatid exchange compared to controls in the study of Livingston et al (1980). In support of these reports of chromosomal effects of asbestos on fibroblasts is the report of Huang (1979) that crocidolite, chrysotile and amosite are all weakly positive in a fibroblast mutagenesis assay; this contrasts with the study of Chamberlain and Tarmy (1977) who found

chrysotile, superfine chrysotile, crocidolite, amosite and anthophyllite, to be negative in Ames type bacterial mutation tests.

Daniel et al (1980) reported that asbestos treatment of foreskin fibroblasts in culture enhanced the binding of polycyclic aromatic hydrocarbons to the fibroblast DNA, an effect which the authors noted had been reported to have a clear relationship with carcinogenic potential.

Biochemical evidence that asbestos alone can produce transformational type changes in fibroblastic cells was provided by Neumann et al (1980). This group reported that Syrian hamster embryo cells treated with asbestos showed simplification of cell surface glycoproteins and glycolipids in a manner reminiscent of the changes following alteration from the normal to the transformed phenotype induced by conventional agents in many previous studies.

Tejwani et al (1980) reported inconclusive results as to the effect of asbestos on the cyclic nucleotide balance of fibroblasts; chemical carcinogens decrease this ratio but asbestos, while lowering the ratio initially, caused doubling of the ratio on longer incubation. The authors noted that this pattern of change was found with some tumour promoters.

1.10.2 Direct effects - epithelial cells

Adding asbestos to an epithelial cell line (monkey kidney) in vitro Mahon and Eckert (1976) showed that all asbestos types, at doses insufficient to impair viability could depress viral interferon induction by up to 90%. A corollary of this was that more rapid viral replication was noted in asbestos treated cells. The important role of viruses in animal carcinogenesis and its postulated but unproven role in human tumours (Pitot 1978) make this finding particularly interesting.

Mossman et al (1980) added chrysotile at very low levels (0.5 µg/ml)

and crocidolite (5 µg/ml) to an epithelial cell line derived from trachea and found increased levels of DNA, presumed to reflect increased proliferation of the epithelial cells.

Hamster trachea organ culture has been utilised (Mossman et al 1977; Frank 1980) and it has been shown to undergo increased basal cell hyperplasia on treatment with asbestos. This is particularly interesting in view of the fact that basal cell hyperplasia has been characterised as a pre-malignant state.

1.10.3 Direct effects - mesothelial cells

In 1972 Rajan et al used explants of human lung to study the effect of crocidolite asbestos on the pleural mesothelium in vitro and found that culturing lung fragments in the presence of crocidolite caused proliferation of the mesothelial cells of the visceral pleura, from a monolayer to 5 or 6 cells thick. This was accompanied by "invasion" by these cells of the underlying lung and increased nuclear size; identical pieces of lung from the same donor grown in the absence of asbestos showed a normal mesothelial monolayer.

It was not until 1978 however that Bignon and co-workers managed to grow a homogeneous mesothelial cell line from scrapes of rat parietal pleura (Thiollet et al 1978) and they were then able to examine the effects of asbestos on this pure population.

This group reported that cultures of mesothelial cells phagocytosed UICC chrysotile with subsequent formation of secondary lysosomes (Jaurand et al 1979b) and that sub-lethal doses of chrysotile caused vacuolation in the cells which eventually recovered but with an increased doubling time, possibly suggesting some damage to the synthetic machinery of the cell (Kaplan et al 1980 a). In their system there was a failure

to demonstrate any difference in rate of sister chromatid exchange between untreated mesothelial cells and those treated with chrysotile (Kaplan et al 1980 b). Bignon and co-workers are in the midst of a conprehensive study of the effects of asbestos on their mesothelial cell line with regard to long term asbestos treatment and mutagenesis testing of asbestos alone and in association with other substances (Kaplan et al 1980 b) and the results of these studies are awaited with interest.

1.10.4 Overview

It is clear from these studies that both the fibrogenic and carcinogenic properties of asbestos can be manifest in experiments on the direct effects of asbestos on fibroblasts, epithelial cells and mesothelial cells in culture. Thus fibroblasts can be induced to proliferate excessively and lay down abnormal collagen in response to asbestos treatment; asbestos above a certain length can also provide a sufficient substratum for anchorage dependent growth. In terms of carcinogenic response chromosomal damage, sister chromatid exchange, biochemical evidence of transformation and increased DNA binding of polycyclic aromatic hydrocarbons have all been reported following asbestos treatment. It is notable that fibroblasts, while obvious candidates for fibrogenic effects, are not traditionally seen as candidates for the carcinogenic effects of asbestos. It can be assumed that their selection as target cells in some of the above studies detecting carcinogenic type effects are a matter of convenience or routine in the assays used. However, asbestos has been detected by electron microscopy in the lung interstitium 24 hours after a 1 hour inhalation exposure to chrysotile in rats (Brody and Hill 1981); close interactions between asbestos and inter-

stitial fibroblasts could therefore be an early sequel to inhalation. Many mesotheliomas are described as fibrosarcomatous and are manifestly fibroblastic in appearance and it seems possible that at least some "mesotheliomas" are actually fibroblastic in origin although the common mesenchymal heritage of both fibroblasts and mesothelial cells makes a judgment on the stem cell of such a tumour difficult.

Evidence that asbestos can directly affect epithelial cells in ways consistent with carcinogenesis is present in the evidence that asbestos inhibits interferon induction and also causes increased proliferation of epithelial cells in a small number of studies. Asbestos can be taken up by Type I alveolar epithelial cells within 1 hour of commencement of inhalation (Brody and Hill 1981) and so the possibility for prolonged interaction exists.

There is little evidence for direct "carcinogenic type" effects of asbestos on mesothelial cells in culture except for data reporting mesothelial proliferation in organ culture and which does not appear to have been repeated by other groups.

1.11 Possible effects of asbestos in fibrosis through its immune and inflammatory effects

The most likely candidates for the indirect effects of asbestos on fibroblasts are the body's major defence systems, the inflammatory and immune systems. These highly complex systems interconnect and overlap so that the response to an inflammatory or immunogenic agent can bring in aspects of both systems. The "final" common pathway of both systems can be fibroblast proliferation as in the case of scar formation when the stimulus is purely inflammatory, e.g. a sterile cut; and in the case of fibroplasia in granulomatous disease of immunological origin, e.g. tuberculosis. In view of the current evidence as to the central role of the macrophage in immune response, inflammatory response and

regulation of fibroblast proliferation (see later) the macrophage appears as the most likely cell to arbitrate between the inflammatory and immune systems and in the final resolution of the reaction by fibroblast proliferation. The major role of the alveolar macrophage in phagocytosis and clearance of inhaled asbestos (Miller 1978) and other fibrogenic dust make it additionally likely that the macrophage could have a major role in the development of pulmonary fibrosis.

It is proposed to review relevant aspects of macrophage function with particular reference to inflammation, immunity and control of cell proliferation and discuss where asbestos might have its effects. Where appropriate other aspects of host defence will be discussed along with the known effects of asbestos.

1.11.1 Macrophage function

1.11.1.1 Macrophages - nomenclature

The term mononuclear phagocyte was introduced to describe the cells of the monocyte/macrophage series (van Furth et al 1972) but the term macrophage will be used here to describe the adherent mononuclear cells of the peritoneal cavity, alveolar spaces and other sites.

Macrophages are present in the mammalian body as 56.4% in the liver (Kupfer cells), 14.9% in the lungs, 7.6% in the peritoneal cavity and 21.1% in other tissue sites (van Furth et al 1980).

1.11.1.2 Ontogeny

The origin of macrophages at various sites has been elucidated in recent years principally through the work of van Furth and his co-workers using mice and rabbits (for example review by van Furth et al 1980). All cells of the macrophage lineage arise from primitive monoblast precursors in the bone marrow, through monoblast, promonocyte

to monocyte. Monocytes enter the peripheral blood where they remain on average for 1 day (Bainton 1980); they then leave the peripheral blood to become tissue macrophages at one of the above mentioned sites. The large number of monocytes in the blood and the fact that they only remain there on average for one day means that macrophages at tissue sites are a constantly renewing population from incoming monocytes.

During an inflammatory response in the murine peritoneal cavity, when there is increased recruitment to that site, a specific factor is present in the serum which induces monocytopoesis (Sluiter et al 1980); towards the end of the acute inflammatory phase this is replaced by a factor inhibiting monocytopoesis. These factors appear to be released by macrophages themselves at the site of inflammation and feed back to the bone marrow acting at the level of monoblast or promonocyte division (van Waarde 1978). Although the bulk of evidence is that turnover of tissue macrophages is met by replacement from blood monocytes there is evidence that local macrophage division may have a role in normal turnover and an increased role at inflammatory foci in the peritoneal cavity (Stewart et al 1975) and lung (Evans et al 1973). Thus up to 10% mitotic macrophages were present in the alveoli of NO_2 exposed lung (Evans et al 1973) and up to 10% of macrophages at an adjuvant induced inflammatory site were synthesising DNA (Spector 1977).

1.11.1.3 Function

The functions of macrophages are now known to be so extensive, and their activities and secretions so numerous, that a complete review is not appropriate here. The functions of macrophages have been extensively reviewed elsewhere with regard to their role in inflammation (Allison et al 1978; Edelson 1980; Davies and Bonney 1980); in the sensory

and effector arms of the immune system (Nelson 1976; Unanue and Rosenthal 1980); modulatory effects on tumour cells (Fink1976; James et al 1977; Keller 1981), and in phagocytosis (Stossel 1976; Walters and Papadimitriou 1978). That one cell can apparently carry out this multiplicity of functions may be explained in part by heterogeneity and activation as discussed below.

1.11.1.4 Heterogeneity

The site related heterogeneity of macrophages is well documented particularly with regard to differences between alveolar and peritoneal macrophages due to the ease of obtaining these populations for comparison (Hopper et al 1979; Walker 1976; Stuart 1977). As well as functional differences there are also differences in expression of specific antigens between alveolar and peritoneal macrophages (Walker 1976) and recently monoclonal antibodies have been used extensively to detect macrophage subsets (see papers in Forster and Landy Eds. 1981). The fact that the vast majority of macrophages at any site are derived from blood monocytes makes it likely that local, environmentally produced differentiation signals are responsible for site specific variation in macrophage phenotype.

As well as showing site specific heterogeneity the population of macrophages at any one site display heterogeneity with regard to the expression of various structural and functional properties. Thus peritoneal macrophages display a range of Fc receptor activities with some cells having high avidity receptors and some having low avidity receptors, as measured by specific rosetting techniques (Rhodes 1975). Similarly the proportion of any macrophage population reacting to anti-macrophage sera shows considerable variation suggesting heterogeneity

in expression of macrophage membrane antigens (Hopper et al 1979; papers in Forster and Landy Eds. 1981). There is also heterogeneity in size within any macrophage population and different sized sub-populations have been shown to possess different functional properties with, for instance, the large macrophage sub-population possessing the most effective anti-tumour potential (Lee 1980).

The multitude of functions and secretions now attributed to macrophages makes this property of heterogeneity within macrophage sub-populations less surprising since only specialisation or the presence of macrophages at different stages of differentiation (activation) could reasonably account for this bewildering spectrum of activity (Hopper et al 1979).

1.11.1.5 Activation

One of the most studied manifestations of normal macrophage function, and one which probably underlies much of the evidence for heterogeneity in macrophage populations, is the change from resting to activated macrophages. The term macrophage activation was first used by Mackaness approximately 20 years ago (for example Mackaness 1964) who described the increased bactericidal activity of macrophages from infected animals. Although initially thought to be a specific response of the macrophage it was later shown that such activated macrophages could kill a variety of microbes. In 1972 Simon and Sheagren demonstrated that control macrophages incubated with sensitized lymphocytes plus antigen attained increased bactericidal potential so demonstrating that the specificity of the response resided in lymphocytes which transferred this non-specific protective function to macrophages. It was also established that lymphokine capable of activating macrophages was generated by lymphocytes responding to allogeneic cells, T cell mitogens and B cell mitogens (North 1981). The fact that animals bearing chronic infection

were resistant to tumour cell inoculation led Hibbs (1972) and others to test whether this resistance was macrophage mediated. When macrophages were found to possess tumouricidal potential, the natural corollary, that macrophages in culture could be activated to the tumouricidal state by lymphokine treatment, was also demonstrated (for example Piessens et al 1975). Macrophages activated with lymphokine have many properties which differ from resting macrophages including altered secretions and altered functions (Morahan 1980) and these will be discussed in detail with regard to the role of the activated macrophage in inflammation, immunity and control of cell proliferation, in later sections.

It has been shown that some, but not all, of the properties of lymphokine activated macrophages are also shared by inflammatory macrophages elicited by inflammatory stimuli in the peritoneal cavity (Karnovsky and Lazdins 1978; Cohn 1978). Such macrophages have been referred to as "non-specifically activated", "non-immunologically activated", stimulated or inflammatory macrophages to distinguish them from lymphokine activated macrophages (Morahan 1980). The most quoted difference between the two types of activation is that inflammatory macrophages are not tumour cell cytotoxic while lymphokine activated macrophages are (Cohn 1978). This is not, however, a hard and fast rule since treatment of macrophages with C3b, a complement cleavage product released during inflammation, renders them tumouricidal (Schorlemmer et al 1977a) and macrophages collected on subcutaneously implanted coverslips, a purely inflammatory stimulus, have been reported to show tumour cell cytotoxicity (Poste 1979). Macrophages activated by both immunological and non-immunological stimuli have many properties in common and this has led Cohn (1978) to postulate a temporal sequence of adoption of properties during activation. The adoption of some of

the early properties in the sequence are seen as pre-requisite in priming the macrophage for the lymphokine signal which raises it to the full tumouricidal state; this is supported by in vitro studies on differentiation signals in activation (Russell et al 1977; Poste 1979; Hibbs et al 1980).

1.11.2 Macrophages and Inflammation

Inflammation is one of the most important homeostatic mechanisms in mammalian functioning and ever since the pioneering work of Metchnikoff (1893) the central role of phagocytic cells in the process has been emphasised. Local tissue injury, due to any invading substance or trauma elicits changes in the microvasculature which culminate in a cellular infiltrate of macrophages, neutrophils and other cells to the inflammatory focus; these act to eliminate the damaging agent by phagocytosis and lead to resolution. The humoral and cellular arms of the immune system may be brought into play in the case of an immunogenic agent and humoral responses may lead to complement mediated lysis or opsonization of micro-organisms which aids in their clearance. The cellular arm of the immune system acts in the case of viruses, parasites and fungi to eliminate altered cells. These specific effectors are joined by the complement, clotting and kinin cascades and other cellular elements in a complex network of interactions which generate a full inflammatory reaction. This may continue as chronic inflammation, or may resolve, depending on the nature of the inflammatory agent.

Macrophages and other cells accumulate at inflammatory foci by chemotaxis, in response to a variety of chemotaxins released at the site of acute inflammation; these include bacterial products, complement components, Hageman factor (Factor XII) and lymphokines (Bainton 1980). Macrophages which accumulate at the inflammatory site become activated and release a large number of inflammatory mediators including acid

hydrolases and neutral proteases, complement components, interferon, endogenous pyrogen, tissue thromboplastin and prostaglandin (Allison et al (1978). It has been noted (Schorlemmer et al 1977b) that many agents which elicit chronic inflammation also activate complement by the alternative pathway to yieldC3b which is a potent macrophage activating agent (Schorlemmer et al 1976). Activated macrophages in turn secrete C3b, C3 and proteases which cleave C3 to C3b and C3a. Thus local alternative pathway activation could initiate an "autostimulatory loop" of C3b mediated macrophage activation. Such activation is non-immunological since it is not T cell mediated. Another example of non-immunological activation occurs when macrophages are activated by agents in decomplemented serum or serum free conditions. In these cases local direct activation of the alternative pathway cannot occur so C3b from this source cannot be a contributory factor to activation. There is however now evidence that agents which can activate under these circumstances (e.g. zymosan, polyanions) can induce macrophages to release C3b which then activates local macrophages to release C3b resulting in a cascade of macrophage activation (Schorlemmer et al 1981). Additionally it has been suggested that binding of some agents to the macrophage membrane, such as lipopolysaccharide (LPS) or Corynebacterium parvum (C. parvum) might cause an increase in turnover of membrane phosphatidylinositol which could cause sufficient perturbation to potentiate the membrane to further stimulation (Weir and Ogmundsdottir 1980; Ogmundsdottir and Weir 1980).

During inflammation due to an immunogenic agent macrophage activation will also occur via lymphokine.

1.11.3 Asbestos and inflammation

The first suggestion that macrophage activation might play a role in asbestos disease was the finding by Davies et al (1974) that treatment

of macrophages in culture with chrysotile induced release of lysosomal enzymes into the supernatant without cell death. Such selective release of lysosomal enzymes by macrophages is induced by a host of other macrophage activating agents both immunological and non-immunological for example, zymosan, dental plaque, streptococcal cell walls, immune complexes, lymphokine and C3b (Davies and Allison 1976; Schorlemmer et al 1981). Shortly after this demonstration of chrysotile induced macrophage activation, Hamilton et al (1976) reported that intra-peritoneal chrysotile and chrysotile treatment of peritoneal macrophages in vitro, both caused increased release of the neutral protease plasminogen activator. The neutral proteases are a group of inducible enzymes whose substrates include the major connective tissue components and so these enzymes may be particularly important in fibrosis and other disorders where cells proliferate beyond their normal confines necessitating remodelling of the connective tissue stroma. Apart from plasminogen activator the neutral proteases include elastase, collagenase and proteoglycan degrading enzyme (Davies and Bonney 1980) and it seems possible that the neutral protease could be particularly important in the chronic inflammation and fibrosis associated with asbestos exposure. Plasminogen activator is an important macrophage secretion since as well as being active in connective tissue destruction by activating collagenase, it also mediates inflammatory processes such as complement activation and the clotting and kinin cascades (Hamilton 1980). Release of neutral proteases can be induced by a large number of macrophage activating agents including thioglycollate, endotoxin, zymosan, Concanavalin A, phorbol ester and lymphokine (Davies and Bonney 1980).

Wilson et al (1977) reported that asbestos could activate complement directly via the alternative pathway when added to cell free, fresh serum; complement cleavage products (probably $C\overline{7a}$ and $C\overline{3a}$)

with chemotactic activity for peripheral blood leukocytes, were also generated. This placed asbestos alongside the other "alternative complement pathway activating" inducers of inflammation discussed above. The ability of asbestos to directly activate the alternative pathway has now been amply confirmed (Hasselbacher 1979; Saint-Remy and Cole 1980) and all 5 UICC samples have been found to have this potential. In addition Saint-Remy and Cole (1980) reported that magnesium leached chrysotile still retained this activity suggesting that chrysotile might reside for a long time in tissue and still retain inflammation generating activity.

The complement system is intricately connected with the other plasma-borne inflammation regulating pathways - the clotting, fibrinolytic and kinin systems, to form a network of interrelated pathways of inflammatory mediation which has been called a "tangled web" (Ryan and Majno 1977). The activation of any of these systems leads to mobilisation of the full inflammatory response. In this regard it is interesting to note that Hamilton et al (1981) have lately shown the amphiboles to be potent activators of Hagemann factor in serum which leads in the first instance to initiation of the kinin cascade. The ability of the amphiboles to activate Hagemann factor places them alongside other negatively charged surfaces such as collagen, articular cartilage, renal basement membrane, sodium urate crystals of gout, pyrophosphate crystals of pseudo-gout, glass and kaolin, which also have this effect (Spragg 1974; Ryan and Majno 1977). The final product of the kinin cascade is kinin (bradykinin) which is a highly potent inflammatory mediator causing smooth muscle contraction, blood vessel dilatation and increased vascular permeability. Other products of the kinin cascade include:- plasmin, which activates the alternative pathway of complement through its C3 cleaving activity; kallikrein, which is a chemoattractant

and which, due to its Hagemann factor activating activity produces a kinin activating "loop"; and plasminogen activator, with the multiple activities discussed above. Hamilton et al (1981) also reported that amphiboles could induce the clotting cascade although this is very likely a direct consequence of their Hagemann factor activating potency.

Prostaglandins represent another group of important inflammatory mediators which have been found to be produced in increased amounts by macrophages treated with asbestos in vitro (Humes et al 1977; Sirois et al 1980). Prostaglandins mediate inflammation in a complex way (Zurier 1974) and while they are almost certainly important in macrophage activation their exact role is not understood (Barlin et al 1981; Bonney et al 1981).

It is clear from the above studies in the cell biology of inflammation and the effects of asbestos, that asbestos is capable of activating the complement/clotting/kinin/fibrinolytic system of plasma in a direct way, possibly mainly through Hagemann factor activation. Asbestos is also capable of generating inflammatory mediators through direct effects and possibly indirect effects (via complement) on macrophages to stimulate the release of lysosomal hydrolases, plasminogen activator and prostaglandin. These experiments, mostly reported in the last 8 years, provide a cellular/molecular basis for the inflammatory generating effects of asbestos which have been realised for many years.

Epithelial damage and inflammation

This discussion of asbestos and inflammation has been limited to the effects of asbestos on the cellular and humoral inflammatory mediators. It is however important to point out that direct epithelial or endothelial damage by asbestos could result in exposure of basement membrane and altered vascular permeability and thereby also lead to inflammation (Pickrell 1981).

1.11.4 Immune responses and macrophages

Macrophages are central cells in the functioning of the immune system in both the sensory and effector arms of both humoral and cell mediated immunity. The immune functions of macrophages will be reviewed with particular reference to macrophage activation, in view of the previously discussed evidence for the macrophage activating potential of asbestos.

1.11.4.1 Antigen presentation

Macrophages take up antigen and process it through their vacuolar system before re-expressing it on their surface membrane as highly immunogenic fragments which are recognised by T and B lymphocytes leading to initiation of immune responses (Pierce and Kapp 1978). This presentation function of macrophages is an absolute requirement for induction of T helper cells in vitro (Erb and Feldmann 1975) and antigen presented in this way can be 1,000-10,000 times more immunogenic than antigen in the soluble form (Mitchison 1969). Antigen is displayed on the macrophage surface in association with Ia antigens which are Ir gene products of the major histocompatibility complex. Simultaneous display of Ia antigen has been found to be mandatory for antigen presentation by macrophages for initiation of the following immune responses:- presentation to B cells for antibody production (Niederhuber et al 1979); antigen presentation to T cells for antigen specific proliferation (Braendstrop et al 1979); generation of cytotoxic T cells to syngeneic tumours (Woodward et al 1979). Requirement for Ia expression results in genetic restriction with regard to T cell/macrophage (Rosenthal and Shevach 1973) and B cell/macrophage (Howie and Feldmann (1978) interaction for induction of immune responses. Ia phenotype is not a stable characteristic in macrophages and reversal of Ia^- to Ia^+ can be elicited by lymphokine treatment in vitro (Steinman et al

1980). Ia expression seems to be a transient event, however, which, once lost by mature macrophages cannot be regained (Beller and Unanue 1981).

A soluble factor released by macrophages which acts on a T cell subpopulation to amplify T helper induction has been found to be Ia bound to a fragment of antigen (Erb and Feldmann 1976) and has been designated Genetically Restricted Factor.

1.11.4.2 Macrophage secretion in immune response

Macrophages secrete a variety of immunomodulatory molecules which have potential to both activate and inhibit lymphocyte function.

(i) Immunostimulatory activity The secretion of non-specific lymphocyte stimulation factors are a major factor in the absolute requirement for macrophages in lymphocyte proliferation in response to antigens, mitogens and heterologous lymphocytes (Wing and Remington 1980). The most studied lymphostimulatory molecule secreted by macrophages is Lymphocyte Activating Factor (Gery et al 1972) also called Mitogenic Protein (Unanue 1978a) and latterly Interleukin 1. Interleukin 1 (IL1) has potent stimulating activity for thymocytes and to a lesser extent T and B cells (Unanue 1978b) and is generated in large amounts when immune T cells, macrophages and antigen are co-cultured (Unanue et al 1976). The fact that non-stimulated and peptone induced macrophages release increased amounts of IL1 following phagocytosis, while thioglycollate induced macrophages are very poor secretors following phagocytosis (Unanue et al 1976), suggests that level of activation exerts a very fine degree of control over synthesis or secretion of the molecule.

Macrophages also release a factor which induces thymocytes to differentiate to the mature T cell phenotype (Beller et al 1978). Macrophages can secrete activity which enhances B cell function in

mice (Calderon et al 1975); this activity causes differentiation of antigen pulsed B cells into antibody producing cells and is T cell independent (Hoffmann et al 1979). A polyclonal B cell activator has also been reported to be released by rabbit macrophages. By contrast this factor acts on T cells to release a soluble product which induces polyclonal immunoglobulin synthesis by primed lymph node lymphocytes (Waldrep and Reese 1981).

(ii) Suppressor activity

Macrophages also release molecules which have potential to inhibit lymphocyte function. Allison (1978) lists 8 products of macrophages which could non-specifically inhibit lymphocyte responses in vitro; these are thymidine, arginase, polyamine oxidase, complement cleavage products, prostaglandins, cAMP and interferon. Most of these substances are present, or are released, in increased amounts by activated macrophages and this goes some way to explaining the substantial literature reporting inhibitory or suppressor effects of macrophages on lymphocytes (reviewed by Nelson et al 1976). Evidence that suppression is increased where macrophages are activated comes from graft versus host disease (Sjoberg 1972), adjuvant stimulation (Klimpel and Henney 1978), infection (Wadee and Rabson 1981) and tumour growth (Herberman et al 1980). A typical example is found in the report of Warner et al (1981) that resting peritoneal exudate macrophages were not appreciably suppressive to PHA mitogenesis of lymphocytes while peptone induced macrophages were.

While suppression may be non-specific and mediated by well characterised cell modulating factors originating from macrophages, such as prostaglandin, it has also been shown that macrophage suppression may be specifically mediated through the agency of soluble factor(s), so far uncharacterised, which activate suppressor T cells (Wadee and Rabson 1981). In contrast Holt et al (1981) using alveolar macrophages,

have reported evidence for a T cell which responds to mitogen by releasing a "suppressor signal" translated by adjacent activated macrophages into a lymphostatic response.

It seems likely that functional heterogeneity particularly in activation level within macrophage populations, could explain the contradictions present in apparently opposing reports of macrophage stimulatory and suppressor activity in vitro discussed above. Thus Holt (1979) has reported functional heterogeneity in rat alveolar macrophage populations where a weakly adherent population supported lymphocyte function and a strongly adherent population suppressed it. Similarly Nelson et al (1976) describes a dialysable inhibitor and a non-dialysable potentiator of lymphocyte mitogenic response, in macrophage supernatants. Differences in experimental technique can also be important in these types of experiment, since ratio of macrophage to lymphocytes (Pennline and Merscowitz 1981) and time in culture before testing for lymphomodulatory activity (Wing and Remington 1980) have both been shown to profoundly affect the expression of suppressor activity by alveolar and peritoneal macrophages.

1.11.5 Experimental studies on asbestos and immune response

Experimental studies on the effects of asbestos on elements of the immune response have been carried out mostly in animal models but human cells have also been used.

1.11.5.1 Cell mediated immunity

Using rats Miller and Kagan (1976) were the first to emphasise the possible effects of asbestos on the immunological role of the alveolar macrophage and to present data suggesting that asbestos exposure in vivo could result in macrophage activation. In early studies altered characteristics of in vivo asbestos exposed macrophages were reported including altered membrane morphology, increased Fc receptor

activity and general activated appearance (Miller and Kagan 1976; Miller et al 1978; Miller 1978); the non-toxic nature of crocidolite, as reflected in the lack of obvious toxic effects on alveolar macrophages from rats inhaling crocidolite, was emphasised, as well as the immunological consequences (Miller 1980). Further studies into the functional potential of asbestos activated alveolar macrophages from rats inhaling crocidolite showed that such macrophages, when cultured with lymphocytes, underwent a close physical interaction with the lymphocytes followed by two separate kinds of lymphocyte proliferation, one of which resembled antigen specific T cell proliferation (Miller et al 1979; Miller and Kagan 1981). This and a previous finding of increased complement mediated immune adherence to these macrophages (Miller and Kagan 1977) was interpreted by the authors as evidence of antigen specific T cell proliferation in response to a putative asbestos related macrophage membrane neo-antigen recognised by sensitised T cells present in dusted rats.

On the basis of their interpretation of these results, the epidemiological data on immune disturbance in asbestotics and sporadic reports of asbestos related lymphoproliferative neoplasms (Lieben)1966; Robinson et al 1979) Kagan and Miller have gone on to suggest a unifying role for uncontrolled lymphocyte proliferation in response to asbestos induced altered macrophage membrane antigenicity, in the pathogenesis of asbestos related disease (Kagan 1980; Kagan and Miller 1981).

Using in vitro asbestos treatment of normal human peripheral blood mononuclear cells (PBMC) Kagamimori et al (1980) reported that chrysotile was not toxic to the lymphocyte fraction but toxic to the monocyte fraction. Decreased PHA blastogenesis of the PBMC was therefore attributed to loss of monocyte co-operation in PHA mitogenesis. The fact that 2-mercapto-ethanol, which substitutes for macrophages in

in vitro lymphocyte responses (Opitz *et al* 1978), did not appear to be included in their culture fluid supports this explanation. Addition of chrysotile directly into assays of macrophage mediated antibody dependent cell mediated cytotoxicity against target Chang cells resulted in inhibition of this function; as suggested by Kagamimori *et al* this was probably due to mechanical interference with macrophage/ target interaction.

Rola-Plesczcynski *et al* (1981) used sheep which had been given intratracheal doses of asbestos and found that unfractionated free airway cells showed increased responsiveness to the mitogens Con A, PHA, Pokeweed mitogen and staphylococcal lysate. The effect was in general dose dependent from 1-16 mg of asbestos. The use of unseparated bronchoalveolar cells present problems of interpretation as to the origin of the stimulus for increased thymidine uptake since the mitogen could act (i) directly in lymphocytes to stimulate them; (ii) on macrophages to cause them to release IL1 which stimulates lymphocytes; (iii) on a T cell sub-population to release macrophage activating lymphokine causing macrophages to act as in (ii). Therefore, while this study shows that there is something different about the free airway cells from dusted sheep lungs compared to controls, it is not clear exactly what the difference is. Further fractionation and characterisation of the free airway cells is required to gain useful information on pulmonary response.

1.11.5.2 Humoral immunity

Virtually no work has been carried out into the role of asbestos in humoral immunity. The disturbance to humoral immunity in asbestotics which has been reviewed previously (1.6.2) is, in general, attributed to disturbances of B cell immunoregulation mediated through altered macrophages and T cells. In one study, however, attempts were made

to raise a rabbit anti-serum to asbestos and asbestos coated with BSA. While the author obtained some qualitative evidence in support of the presence of anti-BSA antibody in appropriate anti-serum, even qualitative data in support of specific anti-asbestos activity was unconvincing (Lukens 1978).

1.11.6 Experimental evidence for the role of the macrophage and immune system in control of fibroblast proliferation

It is proposed to review the literature concerning cellular interaction in fibrogenesis with particular reference to the macrophage; this includes both non-immunological production of macrophage derived factors and also production of such factors by immunocompetent cells such as activated macrophages and lymphocytes.

While early workers in experimental pneumoconiosis emphasised the cytotoxic effect of silica or asbestos on macrophages (Allison et al 1966) and even declared cell death to be mandatory for fibrogenesis (Harington 1974) it is now clear that fibroblast accumulation and increased division can occur in response to secretions from live cells. Thus while factors may well be produced from killed macrophages which stimulate fibroblasts in vitro (as discussed below) it remains to be shown that cell death is a necessary prelude to fibrogenesis in vivo.

Early work showing that an in vitro macrophage cytotoxic particle (quartz) could induce release of a diffusible factor which stimulated fibroblasts to secrete hydroxy-proline was carried out by Heppleston and Styles (1967). This active fraction was produced by treating alveolar macrophages with quartz in vitro, disintegrating them, and collecting the supernatant which was added to fibroblast cultures. This effect of quartz was confirmed by others (see review by Heppleston 1982). Kulonen and co-workers have further characterised this quartz induced fibroblast stimulating factor which apparently acts by inhibiting

RN-ase, so allowing collagen protein specific RNA to persist and be expressed as excess collagen synthesis (Kulonen et al 1980). While much valuable information has been gained using this system, as mentioned above, this approach may have limited relevance to the in vivo fibrogenesis produced either solely by immunological phenomena (Boros 1981) or by non-toxic (in vivo) particulates such as the amphiboles (Miller 1978). There is however an accumulation of literature suggesting that fibroblast stimulating factors are also released by lymphoreticular cells during normal functioning.

The suggestion that living macrophages could be mandatory for fibroblast proliferation came from Leibovich and Ross (1975) who reported that guinea pigs depleted of macrophages with hydrocortisone and anti-macrophage serum had diminished ability to repair wounds. Leibovich and Danon (1980) carried out the converse of this experiment and reported that topical application of the macrophage activating agent glucan caused increased macrophage reaction to a wound and resulted in increased fibroplasia. Leibovich and Ross (1976) also reported that fibroblasts growing very slowly in vitro in medium deficient in platelet derived growth factor (platelet poor plasma serum - PPPS) could be stimulated to divide by the addition of supernatants from macrophages cultured in PPPS. The stimulation of division was greater than that produced by addition of appropriate amounts of PPPS. It was particularly noticeable that macrophages killed by freeze-thawing were only about one half as effective in producing fibroblast stimulating activity and that macrophages induced with oil produced slightly more activity than resting macrophages. Leibovich (1978) further characterised this activity and reported two functionally separate activities - one which caused fibroblasts to divide and another which inhibited tritiated thymidine uptake. The complex nature of

the activating phenomenon was revealed by the fact that only macrophages cultured in PPPS released the fibroblast division stimulating activity while macrophages cultured in PPPS, Foetal Calf Serum, blood serum or bovine serum albumen all released increased amounts of the tritiated thymidine uptake inhibiting activity.

The fact that monocytes are recruited into sites of acute inflammation has prompted investigation into these cells as potential sources of fibroblast stimulating activity. Thus De Lustro et al (1980) detected fibroblast stimulating activity in supernatants from peripheral blood monocytes. Glenn and Ross (1981), using platelet depleted plasma derived serum, failed to detect release of fibroblast stimulating activity from cultured monocytes; however treatment with the potent macrophage activating agents, endotoxin, Con A and zymosan could induce substantial release of fibroblast stimulating activity. It was interesting to note that phagocytosis of SRBC's, opsonized SRBC's, latex beads, or treatment with muramyl dipeptide or n-formyl methionyl peptide failed to induce release of the factor.

The growth factor described by Glenn and Ross also showed activity against smooth muscle cells and is described by the authors as a "mesenchymal cell mitogen". Stimulation of mesenchymal cell proliferation has also been ascribed to a macrophage derived growth factor described by Martin et al (1981); in this study peptone elicited macrophages, following phagocytosis of latex, showed a greater than 6 fold increase in release of the growth factor while treatment with LPS and the tumour promoter phorbol myristate acetate produced 4 and 3.5 fold increases respectively. Wahl et al (1980a) has also reported large increases in thymidine uptake and collagen protein secretion by fibroblasts treated with supernatants from endotoxin activated macrophages.

It is clear from the above that normal macrophages and monocytes

can be found to be releasing fibroblast stimulating activity and that activation can cause a considerable increase in this release. Thus during cell mediated immune responses, activation of macrophages at sites where T lymphocytes are secreting lymphokine (MAF) could lead to release of fibroblast activating factors; in confirmation of this are reports that lymphokine synthesis can be detected in immunological granulomas (Boros 1981). It is, however, also apparent that non-immunological activation, e.g. latex phagocytosis and zymosan, can cause increased release of fibroblast stimulating factor(s).

While the macrophage can be seen as the effector of fibroblast proliferation in response to immunological stimuli, lymphocytes themselves have also been reported to release fibroblast modulating activity. Postlethwaite et al (1976) showed that lymphokine generated in response to antigen or mitogen were chemotactic for fibroblasts while Johnson and Ziff (1976) reported that lymphokine added to fibroblast initiated cell division and collagen production. This was confirmed by Wahl et al (1980b) who reported that antigen specific T cell proliferation generated factor(s) which increased tritiated thymidine uptake by fibroblasts.

The cell mediated immunity to collagen found in some fibrotic lung disease (Kravis et al 1976) would appear to be the ideal condition for persistent lymphokine generation by sensitised T cells and persistent macrophage activation by the lymphokine produced by these T cells; both lymphokine and monokine could combine to produce local concentrations of potent fibroblast stimulating activity. Support for this sequence in some types of pulmonary fibrosis comes, in part, from a study on the functional state of alveolar macrophages obtained by lavage from patients with a variety of inflammatory lung conditions including idiopathic pulmonary fibrosis and sarcoidosis (Allen and Doe 1981).

In this small study the macrophages from the lungs of such patients were highly activated by the criteria of lysosomal enzyme secretion, plasminogen activator release, 5' nucleotidase levels and phagocytosis, compared to controls; activation indices decreased with anti-inflammatory steroid treatment.

Further evidence for immunoregulation of fibroblast activity comes from studies into the disease Progressive Systemic Sclerosis (PSS), a connective tissue disorder typified by widespread fibrosis, auto-immunity and the presence of T lymphocytes sensitised to collagen, amongst other endogenous substances. Using a crossed one way mixed lymphocyte culture of peripheral blood mononuclears from PSS patients and normal subjects, Cathcart and Krakauer (1980) reported a large increase in fibroblast stimulating lymphokine in the supernatants where PSS patients were the responders compared to cultures where normal lymphocytes were the responders. This increased activity was manifest as a 6 times increase in hydroxyproline incorporation in the targets and it was interesting to note that supernatants obtained from cultures where both PSS and normals were the responders had equal amounts of factor which stimulated fibroblast proliferation as measured by thymidine uptake. Thus activated lymphocytes from PSS patients produced lymphokine which caused fibroblasts to produce more collagen in the absence of fibroblast proliferation.

The above mentioned macrophage and lymphocyte derived factors which stimulate fibroblasts are largely uncharacterised but there are well characterised endogenous mitogenic agents - the proteases. Considerable evidence now exists that proteases can stimulate cell division and differentiation in a variety of cells (review by James 1980). Fibroblasts, in particular, have been shown to be capable of stimulation to divide and lose contact inhibition, by treatment with

low doses of protease (Burger 1970; Noonan 1976). Rokosova and Bentley (1980) have reported increased division of fibroblasts following treatment with macrophage lysosomal enzymes at low concentration and in view of the above mentioned association between macrophage activation and protease secretion this finding may be of particular relevance.

1.11.7 Experimental evidence that asbestos can affect fibroblast growth via lymphoreticular cells

The evidence that asbestos can cause macrophages to release fibroblast stimulating activity is mostly indirect and concerns evidence, reviewed above, that asbestos can activate macrophages. Similarly, the evidence that asbestos inhalation results in cell mediated immune responses in the lung, and thereby accumulation of fibroblast activating lymphokine, is circumstantial.

Macrophages Allison et al (1977) and Bateman et al (1980) used diffusion chambers to enclose asbestos alone, or asbestos plus macrophages, behind a membrane which only allowed molecules smaller than the pore size of the enclosing membrane to pass out. On implantation in animals both of these studies revealed increased fibrosis around the chamber in the case of asbestos plus macrophages than with either asbestos or macrophages alone; this suggested that interaction between asbestos and macrophages led to release of a diffusible fibroblast stimulating factor. Recently, however, more direct information has been published (Bitterman et al 1981) in abstract showing that guinea pig alveolar macrophages treated with chrysotile for 4 hours in the presence of serum (a time course unlikely to result in cell death in the presence of serum) released fibroblast stimulating activity into the supernatant. Supernatants from control untreated alveolar macrophages had no effect on fibroblast proliferation but no phagocytosis controls were reported.

Lymphocytes The only indirect evidence that lymphokine, possibly

capable of stimulating fibroblast growth as found by Postlethwaite et al (1976), might be present in the lungs of individuals exposed to asbestos has come from the work of Miller et al (1979). Alveolar macrophage activation was present and the release of immunomodulatory molecules by activated macrophages, as discussed above, could lead to lymphokine generation. The studies of Miller did not, however, involve studying lung lymphocytes or lung lymph node associated lymphocytes for lymphokine production. Based on their studies Miller (1980) suggested that splenic T cells from dusted animals were sensitized to asbestos related membrane neo-antigens in alveolar macrophages exposed to asbestos and that these sensitized lymphocytes underwent antigen specific T cell proliferation on encountering the neo-antigen bearing macrophages. If this occurs in the lungs of asbestos exposed individuals then the fibroblast stimulating activity described by Wahl et al (1980b), in supernatants from cultures of antigen specific T cell proliferation, could be released.

1.12 Effects of macrophages on the growth of tumour cells

The cell modulating effects of asbestos so far described have concerned enhancement and suppression of normal cells, i.e. lymphocytes and mesenchymal cells; however macrophages can, under certain conditions, also kill, inhibit or promote the growth of tumour cells. As pointed out by Keller (1981) there has been a marked emphasis on the inhibiting effects of macrophages in tumour cells due, no doubt, to the currency during the seventies of the "Theory of Immunosurveillance" concerning host defence against tumours (Pitot 1978). In this model, macrophages and certain other cells are envisaged as recognising new clones of transformed cells in tissue and killing them (Adams and Snyderman 1979). The growth promoting effects of macrophages on tumour cells have received little attention (Keller 1981).

1.12.1 Inhibitory effects

The evidence that macrophages can inhibit tumour cells has come from both *in vivo* and *in vitro* studies. The *in vivo* evidence has been listed as:- (i) macrophages constitute a significant proportion of cells in tumours undergoing rejection; (ii) agents that depress or deplete macrophages prolong tumour survival; (iii) agents that stimulate macrophage function promote tumour destruction; (iv) macrophages are obligatory for the destruction of some tumours (Adams and Snyderman 1979).

The *in vitro* evidence that macrophages can kill tumour cells is now overwhelming and has been extensively reviewed (for example Fink 1976; James *et al* 1977; Keller 1981). A low level of spontaneous cytotoxicity is evident in resting unstimulated macrophages from mice (Pels and den Otter 1979) and in rats (Keller 1978); activation of macrophages however, results in a dramatic increase in their cytostatic and cytocidal capability against tumour cells. This activation can be induced *in vivo* by injection of immune adjuvants such as Freunds, or bacterial endotoxin, by injection of heat killed bacteria such as *Corynebacterium parvum* or *Bacille Calmette Guerin* and by infection with protozoa such as *Toxoplasma gondii*. A similar activation is achieved *in vitro* by exposure of macrophages to lymphokine (Piessens *et al* 1975). Lymphokine containing the active principal (Macrophage Activating Factor)Kniep *et al* 1981) is generated by sensitized lymphocytes on exposure to antigen (David *et al* 1976), in mixed lymphocyte culture (Dimitriou et al 1975) and by lymphocyte mitogenesis (Mantovani *et al* 1980). Other arbiters of the immune response such as immunoglobulin (as aggregates and complexes) (Evans and Alexander 1976) and complement components (Ferluga *et al* 1978) can also activate macrophages to non-specific tumour cell cytotoxicity.

A rather heterologous group of non-immunological agents including double standard RNA, endotoxin, pyran co-polymer, Concanavalin A and streptococcal cell walls can activate macrophages in a lymphocyte independent manner; some of these are thought to act via the C3b autostimulatory loop (Ferluga et al 1978; Schorlemmer et al 1981) and/or interferon induction (Schultz et al 1977).

Macrophages obtained by all such means are non-specifically tumour cell cytotoxic and exhibit toxicity against a range of tumour cell targets (Keller 1976).

The attainment of the non-specific tumouricidal state has been found not to be an all or none event but apparently requires a sequence of differentiation signals inducing stepwise expression of properties which may culminate in expression of the fully tumouricidal/microbicidal phenotype (Cohn 1978). Evidence for this comes from the work of Russell et al (1977) and Hibbs et al (1980) who identified 4 activational states, presumably representing a continuum, using inflammatory eliciting agents, lymphokine and LPS responsiveness. It can be assumed that local environmental signals generated by the immune and inflammatory systems directly or indirectly modulate the activational state and tumouricidal function of macrophages in in vivo (Meltzer et al 1979).

In addition to non-specific tumour cell cytotoxic activation of macrophages, specific "arming" of macrophages against particular tumour cell lines has also been reported (Evans and Alexander 1976). Arming requires that lymphocytes, sensitized to tumour cells, interact with normal macrophages; specific growth inhibition of the immunizing tumour cells then occurs on incubation of the armed macrophages with the specific targets with no anti-tumour activity shown against irrelevant tumour lines (Evans and Alexander 1972). A non-lymphokine Specific Macrophage Arming Factor could be detected in the supernatant of co-

cultures of tumour cells, macrophages and tumour antigen immune lymphocytes; specific anti-tumour activity was conferred on control macrophages by treatment with this factor which the authors postulated was a T lymphocyte derived cytophilic antibody-like molecule (Evans et al 1972).

The sequence of biochemical events leading to macrophage killing of tumour cells are not well understood but for full tumouricidal function both binding to target cells and release of cytotoxic agents have been seen as obligatory (Adams et al 1981) although there have been reports of tumour cell killing mediated by soluble products of activated macrophages (Sharma et al 1980). In the case of cytostasis both cell contact and diffusible macrophage products have been reported as inhibitory to tumour cell proliferation (Keller 1981). Undoubtedly, when tumour cells and cytotoxically activated macrophages are co-cultured they undergo close physical interaction (Hanna et al 1976) and suggestions as to the nature of the cytocidal effector molecules include transferred lysosomal contents (particularly proteases) (Hibbs 1974), complement components (principally C3a) (Ferluga et al 1978), hydrogen peroxide (Nathan and Cohn 1981) and arginase (Farran and Nelson 1980). The variation in response of different target tumour cell lines to the same cytotoxically activated macrophages (Hibbs and Granger 1981; Keller 1981) and the heterogeneity in macrophage phenotype in any population, make it possible that several, or all, of these mechanisms could apply depending on the exact nature of the participating cells.

There are no published studies on the effects of asbestos on the tumouricidal function of macrophages.

1.12.2 Growth promoting effects

While the anti-tumour function of macrophages is more frequently described, there is also evidence that macrophages can enhance the growth of neoplastic cells in vivo and in vitro and that the activational

state of the macrophages could be a major factor in determining which effector capacity is expressed (Keller 1977).

By studying the kinetics of host cell infiltrate into experimental tumours Evans (1979) postulated that the presence of host cells, including macrophages, were mandatory for tumour growth. Evans (1976) also demonstrated that tumour cells growing in depleted culture medium to reduce their proliferation increased their rate of proliferation when tumour derived macrophage supernatant was added and that this increase was greater than that produced by adding the same volume of new medium. Keller (1975, 1978) using in vitro assays has shown that, depending on the tumour cell target, either inhibition or enhancement of proliferation can be produced by incubation with macrophages. In vivo derived evidence that macrophages can, under some circumstances, enhance tumour growth comes from occasional reports of tumour growth enhancement in animals treated with well documented macrophage activating agents such as C. parvum (Peters et al 1978) and BCG (Piessens et al 1970). Using Winn assays Gabizon and Trainin (1980) found that thioglycollate induced macrophages and freshly excised chemically induced fibrosarcoma cells, at a ratio of 40:1, produced tumours in a significantly shorter time than the appropriate number of tumour cells alone; the final incidence of tumours was the same.

The concept that macrophage derived growth factors, which enhance tumour cell proliferation might have a role in two stage carcinogenesis has been propounded by Salmon and Hamburger (1978). These workers reported that treatment of mice with oil "promoter" resulted in splenic macrophages which released factor(s) promoting tumour cell growth and colony formation in soft agar. This work was extended by Currie (1981) who used FS6 tumour cells which are platelet derived growth factor (PDGF) dependent for normal proliferation and which do not form colonies

in soft agar. Currie found that FS6 tumour derived macrophage supernatant could replace PDGF for FS6 cell proliferation and that presence of the supernatant also induced FS6 cells to form colonies in soft agar. It was notable that proteose peptone elicited macrophages were as effective in bringing about these effects as FS6 tumour derived macrophages and that the tumour promoter 12-tetradecanoyl-phorbol-13 acetate, present in the anchorage independent growth assay, could induce colony formation in soft agar by FS6 tumour cells. These experiments are of interest when taken with the findings of Frantz et al (1979) who reported that fibroblasts were more responsive to the proliferation enhancing effects of growth factor following treatment with phorbol ester promoter.

The conclusion that can be drawn from these studies is that macrophages could be responsible for the growth of some tumours in vivo through the secretion of growth factor-like molecules and that macrophages could be involved as promoters, via the same molecules, in two stage carcinogenesis. There is evidence that phorbol ester tumour promoters can induce some degree of macrophage activation as measured by spreading and phagocytosis (Phaire-Washington et al 1980; Laskin et al 1980) but that they can block lymphokine mediated activation to the tumouricidal state in vitro (Keller et al 1982). It is tempting on the basis of these results to postulate that tumour promoters could operate at (at least) two levels in bringing about promotion:-

(a) macrophages are activated by the promoter sufficiently to release increased amounts of the endogenous macrophage derived growth factor but with blockage of the capacity to become tumouricidally activated;

(b) fibroblasts are affected by the promoter to proliferate excessively in response to the macrophage derived growth factors.

The proven tumour promoting effects of asbestos described earlier

and the obvious fibroblast stimulating effects of asbestos manifest in fibrosis, make these findings of particular relevance in asbestos related neoplasia.

1.13 Central role of the macrophage membrane

1.13.1 Cell membrane structure

Up until the early nineteen-sixties the cell membrane was envisaged as a rather rigid tri-laminar sheath which functioned mainly as a semi-permeable barrier to the extra-cellular environment. On the basis of evolving evidence as to the fluidity of biological membranes and the lateral movement (translational mobility) of membrane proteins Singer and Nicolson (1972) advanced the "fluid mosaic" model of membrane structure. The concept of membrane fluidity and its consequences have been a major force in cell biology and has led directly to the current concept of the cell membrane as a dynamic entity functioning as the principal sensor and effector organelle in cellular control of growth, division, development, communication, differentiation and death (Nicolson 1976). The implications of the fluid mosaic model have been extensively reviewed elsewhere with regard to cells in general (Nicolson 1976a) and with regard to macrophages in particular (Oliver and Berlin 1976). A major tenet of this model is that some of the integral membrane proteins span a membrane lipid bi-layer with a fluidity similar to light machine oil (Nicolson 1976). These trans-membrane glycoproteins have carbohydrate containing moieties displayed at the outer face which may function as specific receptors by virtue of their chemistry and three dimensional shape while at the inner face the protein has the capacity to interact with the contractile proteins of the cytoskeleton (Nicolson and Poste 1976). When this occurs the integral protein may have its free diffusion in the fluid bi-layer affected and

be immobilised, or it may be redistributed in the plane of the membrane in an energy dependent way.

The macrophage, in view of its central position in the immune system and inflammatory system and its endocytic activity, might be predicted to have a particularly highly evolved cell membrane in order to fulfil its multiple functions and indeed the macrophage has been found to have a large complement of receptors and specialised membrane functions.

1.13.2 Phagocytosis

Phagocytosis is a major membrane related process which is carried out by only a few cells and is a particular function of macrophages. Phagocytosis is a complex process involving uptake of a particle following non-specific or specific (receptor mediated) interaction between the particle and the macrophage membrane; this results in local mobilisation of cytoskeletal elements leading to internalization of the particle into a phagosome "pinched off" from the cell membrane. Phagocytosis is an important function of macrophages and is carried out at sites of inflammation where bacteria and cell debris are removed leading to resolution of the inflammatory focus.

Phagocytosis can be seen as a particularly important function of alveolar macrophages since the lung is constantly challenged with organic and inorganic particulates, although a large proportion of this inhaled dust is cleared by the mucociliary escalator, never reaching the alveolar macrophages.

Phagocytosis can be Fc or C3 receptor mediated via immunoglobulin or complement opsonins on the surface of the particles (Michl and Silverstein 1978). Phagocytosis can also be mediated by non-specific receptors for particles such as latex beads, silica or asbestos, which are readily ingested; in these cases phagocytosis is thought to be

mediated by adhesion forces, between the particle and the macrophage membrane glycoproteins, such as electrostatic forces, van der Waal's forces and co-valent bonding (Jones 1975). Once initiated phagocytosis proceeds, in keeping with the "zipper" model, by the membrane interacting sequentially with ligands on the particle surface until the particle is eventually surrounded by membrane and is effectively internalized (Griffin et al 1975a). While the exact nature of the transmembrane signal following contact between the particle and the membrane, which initiates the cytoskeletal mobilisation associated with phagocytosis, is not understood, a general model has been put forward (Michl and Silverstein 1978). In this model interaction between the particle and the membrane receptor results in activation of a "receptor coupling factor" which combines with the cytoskeletal contractile proteins leading to pseudopodal extension and phagocytosis. It is notable that whether the particle attaches via a specific receptor or non-specifically interacts with the membrane the final pathway of cytoskeletal mobilisation, leading to phagocytosis, is the same.

There is evidence with regard to phagocytosis that the macrophage membrane is a mosaic with specialised membrane domains which selectively internalize and discriminate for certain particles via receptor mediated recognition (Griffin and Silverstein 1974); there is also evidence that specialised "transport" areas of the membrane are never internalized at phagocytosis but are selectively excluded (Tsan and Berlin 1971).

1.13.3 Macrophage membrane receptors

1.13.3.1 Non-specific receptors

Macrophages bind a variety of particles via different non-specific binding mechanisms as shown in the study of Benoliel et al (1980), who altered the degree of binding of various particles by altering

the physicochemical conditions of binding; this study also revealed that binding in some cases triggered phagocytosis and in some cases did not. There is also evidence that lectin-like receptors are present in macrophage membranes which play a role in recognising micro-organisms and binding them via their cell wall carbohydrates prior to phagocytosis (Weir and Ogmundsdottir 1977); it is suggested by these authors that this represents an evolutionary primitive recognition system predating the immune system and specific opsonization.

Lectins are plant proteins with high specific binding activity for certain sugar groups and they have been used extensively to study the cell surface oligosaccharides which comprise the carbohydrate moiety of the integral and peripheral membrane glycoproteins. Lectins have been used as probes to study different aspects of membrane structure and function (Nicolson 1976b)as well as tools to induce transmembrane signals such as mitogenesis (see for example Oppenheim and Rosenstreich 1976). Several lectins have been used as probes of macrophage membrane including Concanavalin A (Oliver and Berlin 1976); Lotus tetragonolobus lectin (Homma et al 1981); wheat germ agglutinin (Hadley et al 1977); Phytohaemagglutinin and Pokeweed mitogen (Loor and Roelants 1974). The Concanavalin A receptor which recognizes ∝D glycosyl residues has been particularly used in studies on macrophages with regard to the effect of spreading and phagocytosis (Lutton 1973), ligand induced capping (Williams et al 1979) and transmembrane organization (Adachi et al 1980).

As well as possessing these non-specific receptors macrophages also possess specific membrane receptors for key molecules.

1.13.3.2 Fc receptor

The macrophage receptor for the Fc portion of immunoglobulin is important in the immune phagocytosis of opsonized particles and immune

complexes. Receptors displaying Ig class and subclass specificity have been described. Mouse macrophages possess a trypsin sensitive receptor for aggregated or monomeric IgG2a and a tryspin insensitive receptor for IgG1 and IgG2 in monomeric or aggregated form (Zuckerman and Douglas 1979). Although rats have been described as having an IgE receptor (Boltz-Nitelescu and Spiegelberg 1981) this has not been described in mouse macrophages. The rabbit alveolar macrophage has been calculated to have 10^6 receptors for IgG in the resting state and $2x10^6$ in the activated state (Arend and Mannik 1973). Increased numbers of Fc receptors, as measured by rosetting techniques, have also been described in activated peritoneal macrophages compared to resting peritoneal macrophages (Rhodes 1975). As well as having a role in recognizing opsonized particles the macrophage Fc receptor can also mediate target cell cytolysis and may also aid in lymphocyte macrophage interactions (Zuckerman and Douglas 1979).

1.13.3.3 C3 receptor

Macrophages possess two classes of receptor which recognize C3 cleavage fragments:- type CR1 which recognizes C3b, C4b and C5b components and CR3 receptors which react with C3b1 (Ross 1981). C3 and Fc receptors apparently act in a co-operative manner to mediate immune phagocytosis of opsonized particles since less antibody is required for internalization in the presence of complement (Griffin et al 1975b). Conversely, studies with mouse peritoneal macrophages have revealed that in resting macrophages the C3 receptor mediates binding but not phagocytosis of a C3 opsonized particle; addition of a small amount of specific Ig then results in ingestion (Mantovani et al 1972). Activated macrophages, however, can ingest particles via their C3 receptor alone.

1.13.3.4 MIF receptor

Macrophages possess a trypsin sensitive receptor for migration inhibition factor (David and Remold 1976).

1.13.3.5 Chemotactic factor receptor

Macrophages possess a specific membrane receptor for the synthetic chemotactic agent f.Met-Leu-Phe; the numbers of these receptors are not increased in inflammatory (activated) macrophages (Snyderman and Fudman 1980).

1.13.3.6 Receptor mediated endocytosis

A number of physiologically important molecules are internalized by the process of receptor mediated endocytosis including α_2 macroglobulin, lysosomal enzymes, low density lipoprotein, transcobalamin and hormones such as epidermal growth factor (Stahl et al 1980). Receptor mediated endocytosis involves binding of molecules to specific receptors which are aggregated in specialized membrane areas called coated pits; following internalization of these areas as coated vesicles the receptors may be re-cycled to the membrane (Goldstein et al 1979).

1.13.4 Macrophage membrane associated cytoskeletal components

Cytoskeletal components which are associated with the macrophage membrane integral glycoproteins have been shown to be organised, at areas of macrophage attachment to the substratum, as a web of micro-filaments adjacent to the plasma membrane while next to this area a zone of microtubules which occasionally extended into the body of the cell; the same pattern was seen in regions of membrane in contact with phagocytosable particles (Reaven and Axline 1973). Evidence that these cytoskeletal conponents are indeed important in macrophage membrane related functions, as predicted from the fluid mosaic model, comes from the use of cytoskeleton disrupting drugs; these have been found to

affect locomotion and spreading (Cheung et al 1978); chemotaxis (Rinehart and Boulivare 1977); pinocytosis and phagocytosis (Axline and Reaven 1974); Fc receptor function (Atkinson and Parker 1977) and lectin receptor mobility (Pick and Wilner 1979).

1.13.5 Membrane fusion

A particular property of macrophage membranes is that macrophages can fuse together to form multi-nucleate giant cells or macrophage polykarya under particular conditions. This occurs at inflammatory sites such as granulomas due to chronic infection by bacteria, fungal pathogens, viruses and at sites of foreign body implantation (Papadimitriou and Walters 1979). Immunological factors appear to play a role in membrane fusion as shown by the findings that lymphokine from antigen stimulated sensitized lymph node cells (Galindo et al 1974) and lung washings from animals undergoing immune response to BCG (Parks and Weiser 1975) are able to promote macrophage fusion. Of particular interest here are reports of multinucleate giant cell formation in response to asbestos (Davis 1970b; Sethi et al 1971; Miller 1978).

The foregoing introduction has been aimed at reviewing evidence for a causal relationship between asbestos exposure and lung disease. Experimental evidence that asbestos can directly affect relevant target cells has also been reviewed but emphasis has been placed on the possible key role of the macrophage due to its position in dealing with inhaled particles and its multiple effector functions. In reviewing evidence for the importance of macrophage activation and the central role of the macrophage membrane in macrophage responses. it is hoped that a study of the effects of asbestos on macrophage activation, with special reference to the membrane, is therefore justified.

AIMS AND SCOPE OF THE STUDY

Aims and scope of the study

The foregoing literature review has shown that from the middle 1970's there has been a small number of publications with emphasis away from the lethal effects of asbestos on macrophages; previous studies of asbestos/macrophage interaction had concentrated almost exclusively on the ability of asbestos to kill macrophages. The general aim of this present study, begun in 1979, was to confirm and extend these studies of Hamilton et al (1976) and Miller (review 1978) which first emphasised the non-toxic effects of asbestos in vivo and the possible consequences of asbestos induced modulation of macrophage function in the aetiology of asbestos related disease.

An intraperitoneal asbestos injection model in CBA/Ca mice was chosen and parameters of the induced macrophage population were studied with appropriate controls.

The aims of the study were, in the first instance -

(a) to determine whether ip asbestos injection resulted in the induction into the peritoneal cavity of a macrophage population altered in ways suggestive of macrophage activation,

(b) to determine whether activation to the fully activated tumoricidal state was attained.

Following on from these initial studies which revealed the asbestos induced macrophages to be activated but not tumoricidally activated in an in vitro assay, the study then proceeded along three separate lines.

(a) an examination of the possible consequences of asbestos induced macrophage activation for tumour growth in vivo and in vitro and for immune response in vitro as measured by lymphocyte mitogenesis.

(b) the use of the lectin Concanavalin A as a probe to study asbestos/ macrophage interaction and their consequences for macrophage membrane.

(c) an investigation into the possible expression of a membrane neo-antigen in asbestos activated peritoneal macrophages and its possible consequences in terms of autoimmune humoral response.

Separately from the main macrophage study the biology of tumours derived from the peritoneal cavity of long term asbestos bearing mice was studied to a limited degree.

Through these studies it was hoped to confirm the macrophage activating potential of asbestos for peritoneal macrophages. It was also hoped to gain some information on the possible consequences of such activation for systems in which macrophages are important factors, i.e. tumour growth and lymphocyte function, and which are relevant to the development of asbestos related disease.

MATERIALS AND METHODS

2.1 The model

Choice of a model for studying the effects of asbestos on macrophages. The principal aim of this study was to examine the effects of asbestos on macrophages with special reference to changes induced in the cell membrane. There are several strategies which can be adopted in such a study:-

1. Macrophages can be cultivated, asbestos added and the effects measured.
2. experimental animals can be exposed to clouds of airborne asbestos by inhalation; the alveolar macrophages can then be harvested and studied.
3. experimental animals can have asbestos in solution instilled, via the trachea, into the lungs; the induced macrophages can then be harvested and studied.
4. asbestos can be introduced into the peritoneal cavity of experimental aminals and the induced macrophages harvested and studied.

Strategy 1 carries with it all the drawbacks present in studies where cells are treated in vitro with an agent and the effects measured. This problem is particularly severe with macrophages which are intimately involved in the major body defence system, i.e. the inflammatory and immune systems. It is perilous therefore to make generalisations from such studies with regard to the effect of an agent on macrophages deprived of the multitude of cellular and humoral factors with which they interact in vivo. Strategy 2 is technically and financially beyond the means of this study but is the ideal strategy. Strategy 3 requires the use of rats if non-surgical intratracheal instillation of asbestos is to be carried out. In mice there is a surgical procedure but it carries with it the risk of infection. Strategy 4 was adopted in this study for the

following reasons.

(1) the peritoneal macrophages encounter asbestos in the body where the normal cell and tissue interactive processes can occur.

(2) in particular the process of cell recruitment, replacement and turnover are operative, thus cells recruited into the peritoneal cavity as part of the overall response will also be harvested and studied.

(3) administration to mice by intraperitoneal injection is simple and straightforward for one person as is harvesting of the peritoneal macrophage population.

The major drawback of this model is the well documented difference between peritoneal and alveolar macrophages with regard to some properties (see later). There are, however, many similarities in structure and function and both are derived from monocytes.

2.1.1 Choice of asbestos types

Asbestos can be divided, on the basis of mineralogical differences, into two distinct types (see previous), (a) serpentine (b) amphibole. Chrysotile is the only serpentine asbestos type and so this was used in the study; chrysotile is also the major industrially utilised asbestos type. There are 5 amphibole types with crocidolite as the most industrially important and so crocidolite was also used. In both cases the standard UICC samples were used as these have been prepared to enable cross-laboratory comparison of studies on asbestos. Fig. 2.1 shows the light and electron microscopic appearance of the UICC samples of crocidolite and chrysotile with the latex control particulate for comparison.

2.1.2 Choice of asbestos dose

The choice of a dose for injection into the mouse peritoneum was based on the study by Wirth (1975) who injected UICC asbestos intraperitoneally into mice. Wirth injected 5 mg of asbestos and reported

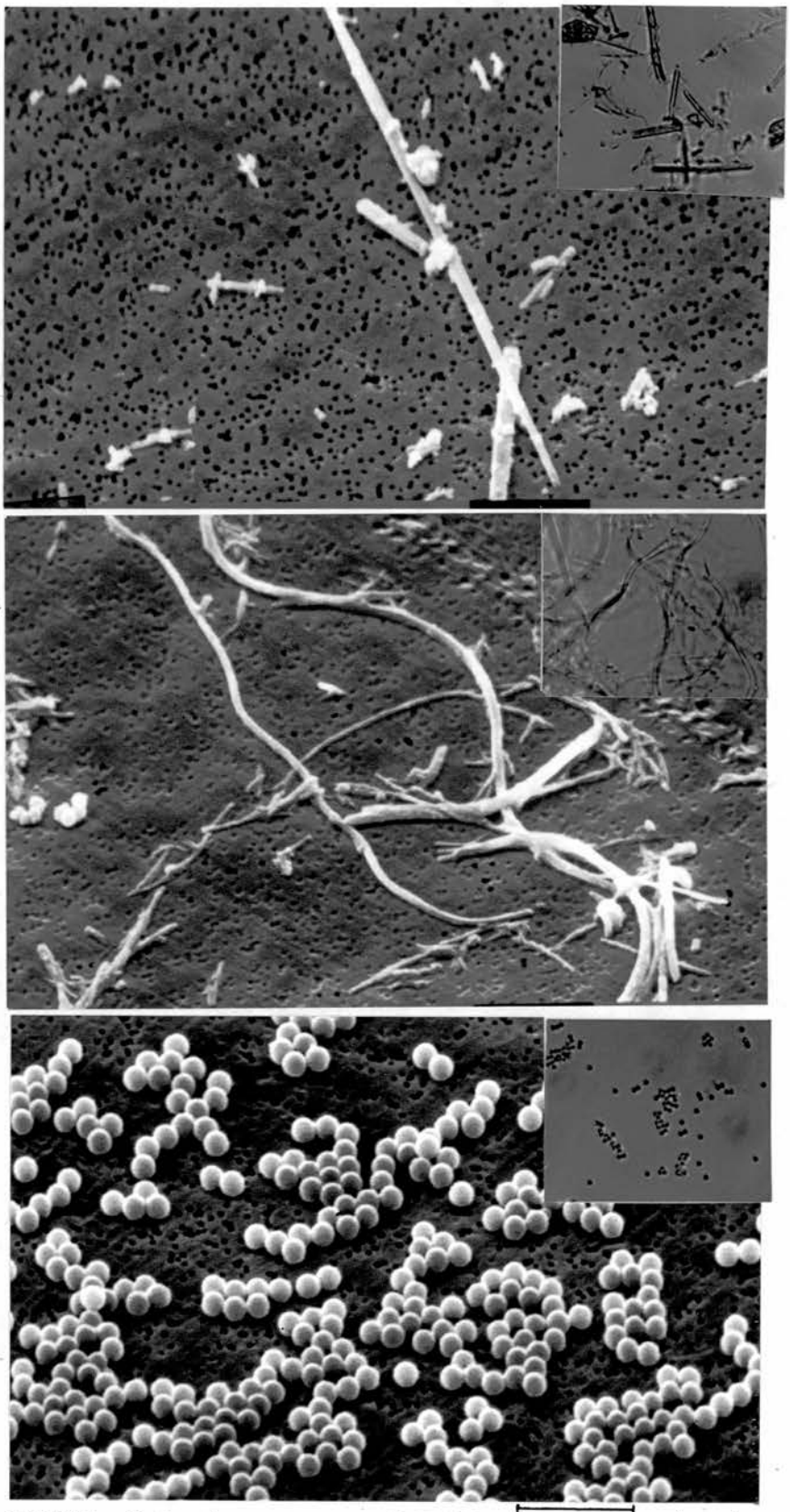


FIGURE 2.1 Appearance of UICC crocidolite (top) UICC chrysotile (middle) and 0.81 μm diameter latex by scanning electron microscopy and high power light microscopy.
Marker line 4 μm

Inset Mag. x 840

fibrotic nodules and a mesothelioma-like tumour developing within 12 months. This dose was therefore used in some early experiments and macrophage activation was found; the dose was arbitrarily halved and macrophage activation was still present so this dose of 2.5 mg was used thereafter. As discussed later a dose of 2.5 mg in the CBA/Ca mice used in this study, proved to be capable of inducing a fibrotic and carcinogenic response.

2.1.3 Choice of controls

Throughout this study three controls have been used -

(a) Dulbecco A Since asbestos was, of necessity, injected into the peritoneal cavity as a solution in sterile Dulbecco A, then sterile Dul A alone was included as a control.

(b) Latex spheres The lung is exposed continuously to inhaled particulates from the environment and the alveolar macrophages constantly phagocytose these particles; the majority of these particles are non-fibrous being of vegetable origin (pollen etc.) or amorphous dust. As a control for any effects on the peritoneal macrophage of a sudden challenge of phagocytosable particles in the peritoneal cavity, spherical, non-toxic latex particles were utilised.

(c) Corynebacterium parvum (C. parvum) The preliminary work in this study indicated that the macrophages induced by intraperitoneal injection of asbestos were activated in some way. C. parvum is a well documented macrophage activating agent in vivo and so 5 day C. parvum induced macrophages were often included as a positively activated macrophage population for comparison with the asbestos activated macrophages.

2.2 Mice

CBA/Ca male mice, originally obtained from the MRC Breeding Unit Carshalton and inbred at the Department of Surgery, University of Edinburgh

and 12-16 weeks old at the time of injection were used throughout this study. They were housed, before and after treatment, in plastic cages with wood shaving bedding. They were fed laboratory mouse diet (McGregor) and tap water ad libitum. The animal house was conventionally ventilated and illuminated on a 10 hour photoperiod at an average temperature of 22°C.

2.3 Intraperitoneal (ip) injection

For ip injection the UICC asbestos samples were prepared at 2.5 mg/ 0.5 ml in sterile Dul A and shaken vigourously prior to injection; latex (1%) (Bacto-latex 0.81 μm diameter Difco) was made up in sterile Dul A; sterile Dul A was also used. Animals were injected with 0.5 ml in the mid-peritoneal cavity beneath the line of the liver and above the bladder. Mice therefore received (i) 0.5 ml Dul A/2.5 mg UICC crocidolite or chrysotile; (ii) 0.5 ml Dul A/1% latex spheres; (iii) 0.5 ml Dul A. In experiments where C. parvum activated macrophages were required 0.2 ml (1.4 mg) of heat killed C. parvum (Wellcome) were injected.

Deaths in the few days following injection and presumed to be due to mis-injection were very rare.

2.4 Harvesting of peritoneal exudate cells (PEC)

When required, mice were killed by ether overdose and the PEC harvested. Three consecutive 2 ml volumes of cold sterile RPMI 1640 containing 10 U/ml Heparin (Pularin) were injected. After each injection the cavity was massaged gently before withdrawing the fluid; invariably 5 ml of fluid plus PEC were retrieved in total from each mouse. The peritoneal washouts from separate mice, containing the macrophages to be studied, were at all times held in separate 10 ml glass conical tubes which were siliconised with 2 coats of Sigmacote (Sigma). Where large numbers of mice were to be harvested, and the PEC could be pooled, the peritoneal washout fluids were collected in siliconised glass universals.

PEC were then centrifuged and washed 3 times with Dulbecco A before being brought into 1-5 ml volumes of complete RPMI 1640 (cRPMI) (RPMI 1640) (Gibco) containing 10% Foetal Calf Serum (Gibco); 1 mM L-glutamine; 50 U/ml penicillin and streptomycin (Gibco) and 25 mM Hepes buffer. PEC were then counted by suitable dilutions in a 0.05% solution of Nigrosin dye in a haemocytometer; viability as shown by dye exclusion was always >90%. PEC were then diluted appropriately in cRPMI for subsequent use in assays.

2.5 Effect of ip asbestos on some properties of the induced macrophage population in the short, medium and long term

2.5.1 Background

In order to gain initial information on the effect of ip asbestos on the induced peritoneal macrophage population, groups of 3 mice were injected ip with asbestos or control solutions and kept for 3, 18 or 70 days before being killed and some characteristics of the peritoneal macrophages determined. These time points were arbitrarily chosen to represent short, medium and long term response. The experiments were carried out twice and the results pooled.

2.5.2 Coverslip culture

All subsequent assays, except for 5' nucleotidase, were carried out on macrophage coverslip cultures prepared in the following manner. One hundred microlitres of PEC at $1x10^6$/ml in cRPMI (obtained as given in 2.4) were pipetted as a drop on to chemically clean, distilled water rinsed, 6x22 mm glass coverslips (Chance Propper) on specially made racks. The coverslips were then incubated in sealed plastic boxes, gassed with 5% CO_2 for 1 hour to allow the macrophages to adhere, then washed vigorously in phosphate buffered saline (PBS) to remove the non-adherent cells.

The following assays were carried out either immediately after the 1 hour incubation, as in the case of spreading assays, or within 4 hours during which time the macrophage coverslip cultures were maintained at 37°C in 5% CO_2.

2.5.3 Phagocytosis assay

Macrophage coverslip cultures had 100 µl of 1% latex spheres (Bacto-latex 0.81 µm diameter Difco) in cRPMI added and incubated at 37°C in 5% CO_2 for 1.5 hours. Coverslips were then washed vigorously to remove the non-phagocytosed latex particles and inverted on a microscope slide on a drop of toluidine blue. Macrophages with more than 1 internalised latex particle, as revealed by a x40 objective lens, were scored as phagocytosis positive and the percentage of phagocytosis positive macrophages in 200 cells was expressed as a percentage for each coverslip culture.

2.5.4 Spreading assay

Immediately the 1 hour of incubation to allow adherence was over, coverslip cultures were washed vigorously in PBS and immersed in May-Grunwald solution and a May-Grunwald Giemsa stain carried out. The percentage of spread macrophages in 200 cells was assessed for each coverslip; an unspread macrophage was scored as one where no rim of cytoplasm could be clearly seen. Fig.3.7 shows examples of spread and unspread macrophages.

2.5.5 Fc receptor avidity assay

(i) Sheep red blood cells (SRBC's) SRBC's (Oxoid) were washed 3 times with Dulbecco A and adjusted to 5% packed cell volume.

(ii) Rabbit anti-serum to SRBC's Anti-SRBC serum which had been raised in New Zealand White rabbits by hyperimmunization and stored at -20°C was used. In preliminary experiments anti-SRBC was prepared at 1/10, 1/100, 1/1000 and 1/10,000 dilutions in RPMI. Similar dilutions of normal rabbit serum (NRS) were included as controls for spontaneous rosette formation.

(iii) Coating of SRBC's with anti-SRBC 0.5 ml of 5% washed SRBC and 0.5 ml of the various dilutions of anti-serum and NRS were incubated

at 37°C for 30 minutes, washed 3 times with PBS and adjusted to 1% in RPMI.

(iv) Fc rosette assay Coverslip cultures had 100 µl of coated SRBC added and left at room temperature for 20 minutes. Coverslips were then washed according to a strictly adhered-to wash procedure and inverted on a drop of toluidine blue. Two or more adherent SRBC's was taken to constitute a rosette and the percentage of rosette forming cells in 200 macrophages was assessed for each dilution of sensitising anti-SRBC and for NRS controls.

(v) Preliminary experiments

Control, 3 day saline, 3 day chrysotile and 5 day C. parvum induced macrophages were tested for percentage rosette forming cells against the panel of variously sensitized SRBC's. Fig.2.2shows that C. parvum and chrysotile induced macrophages had greatly increased Fc receptor avidity as shown by their increased ability to form rosettes at 1/100 and 1/1000 dilutions of sensitizing antibody. It was evident that low dilutions of sensitizing antibody could detect differences in affinity of Fc receptors between saline induced and both crocidolite and C. parvum activated macrophages. This effect was consistently obtained in preliminary experiments and so rosette forming ability with SRBC's coated with 1/1000 dilution of anti-SRBC serum was taken as a marker for high avidity Fc receptor bearing cells.

Thereafter coverslip cultures for each condition at each time point were treated with 1/10 sensitized SRBC's to detect total rosette forming cells, and 1/1000 sensitized SRBC's to detect cells with high avidity receptors.

Spontaneous rosette formation detected with SRBC's coated with various dilutions of NRS were, on average, 7.6 ± 2.2 ($\bar{x} \pm$ sd) for all dilutions of NRS.

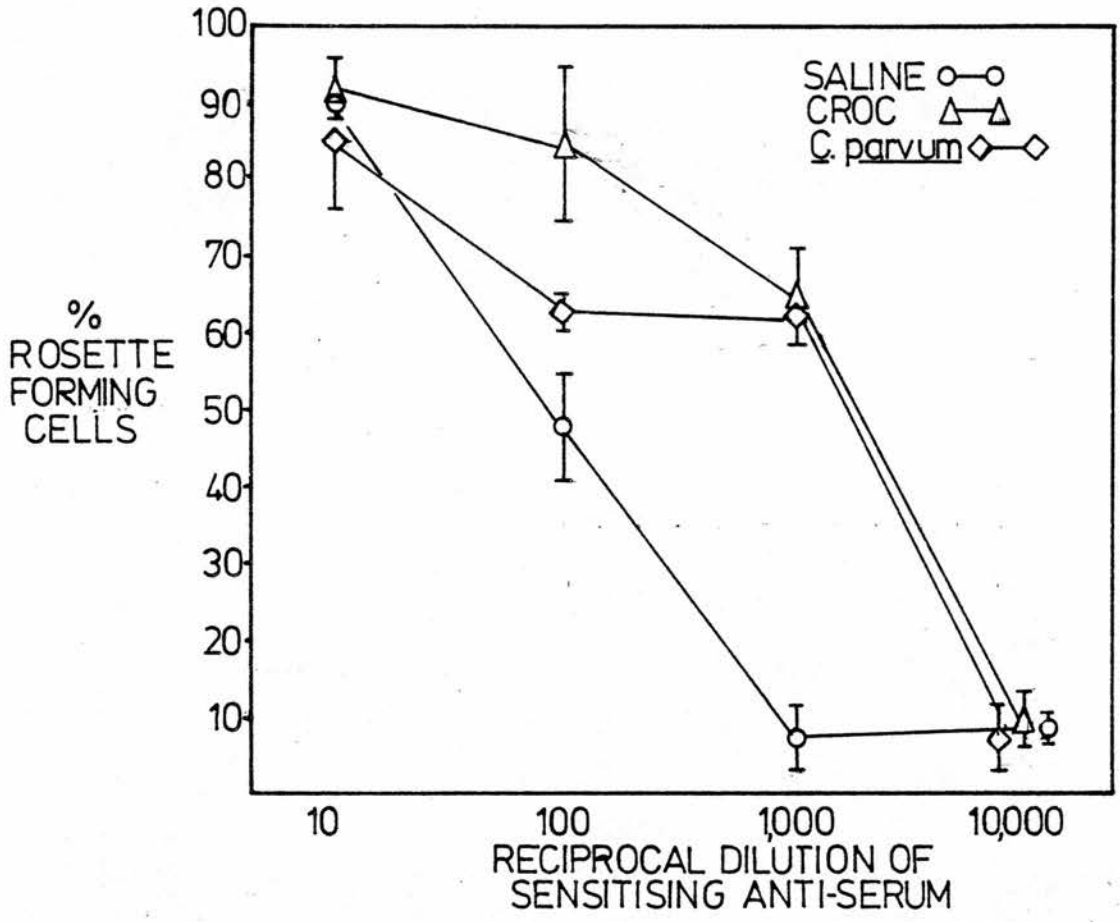


FIGURE 2.2 Mean percentage of macrophages forming rosettes with 1/10 - 1/10,000 dilutions of anti-sheep red blood cell serum. Macrophages induced with saline, crocidolite and C. parvum.
Bars denote $\pm$ one standard deviation.
TRIPLICATE COVERSLIPS (REPRESENTATIVE EXP.)

(vi) Comments on the Fc receptor avidity assay

It was noted that consistent results could only be obtained in this assay if a standardised washing procedure was rigidly adhered to. During washing, coverslips may be particularly prone to shear force which can dislodge Fc receptor bound SRBC's; whatever the reason, the adoption of a standard washing method was a necessity in order to obtain reproducible results.

The source of SRBC's was also a potentially confounding factor in this assay. On one occasion, freshly obtained (same day) SRBC's were used due to failure of the usual source; such SRBC's yielded results entirely different to those routinely obtained with Oxoid SRBC's up to 4 weeks old. The major effect was an approximate 5-fold increase in the apparent avidity of control macrophage Fc receptors; this effect was never obtained with any of the Oxoid SRBC's.

2.5.6 Electron microscopy

In order to obtain information on the morphology of macrophages, transmission and scanning electron microscopy of PEC was carried out. Occasionally electron microscopy was used to study other cells and the procedure was essentially the same as for PEC.

(i) Transmission electron microscopy (TEM)

PEC were washed, centrifuged and resuspended in 4% glutaraldehyde in 0.2 M cacodylate buffer pH 7.2. After 1 hour the cells were centrifuged again and washed 3 times with cacodylate buffer before immersion for 1 hour in 1% osmium tetroxide in 0.1 M cacodylate buffer pH 7.2. Cells were then washed a further 3 times with buffer and processed through graded alcohols before embedding in araldite. Sections were cut on an ultramicrotome (Reichert), mounted on copper grids and stained with uranyl acetate and lead citrate. Sections were viewed and photographed in a Cora (Kratos) electron microscope.

(ii) Scanning electron microscopy (SEM)

PEC were allowed to adhere to 6x22mm glass coverslips for 1 hour before being washed to remove non-adherent cells. Coverslips were then immersed in 4% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 1 hour followed by washing in buffer. The cultures were fixed for 1 hour in 1% osmium tetroxide in 0.1 M cacodylate buffer pH 7.2 and then washed 3 times with buffer prior to critical point drying.

Critical point drying was carried out in a Polaron critical point drying apparatus according to the maker's instructions. Coverslips were brought to absolute acetone by placing in 30% aqueous acetone in a glass tube which was then sealed in a bell jar containing 500 ml of absolute acetone and a beaker of anhydrous calcium chloride; after 2-3 days the 30% acetone was replaced by absolute acetone and the water was taken up by the calcium chloride. Coverslips were then placed in the special "boat" in absolute acetone and placed inside the critical point drier, pressure and temperature were then increased, in accordance with the maker's instructions, until the critical point was reached and then returned to atmospheric pressure. Coverslips were coated with gold in a Polaron sputter coater before viewing and photographing in a Cambridge 600B scanning electron microscope.

2.5.7 Macrophage plasma membrane 5' nucleotidase and lysosomal acid phosphatase assays.

The method used to measure the ecto-enzyme 5' nucleotidase was essentially that of Raz et al (1977).

(i) Enzyme substrate incubation

PEC was adjusted to 10^6/ml in Dul A, 1 ml placed in wells of 24 well plates (Falcon 3008) and allowed to adhere for 1 hour. Wells were then washed 3 times with 1 ml of Dul A and 0.6 ml of distilled water was added; plates were then frozen at -20°C overnight. After thawing 0.6 ml of each

of the following substrates was added to triplicate wells -

(i) 30 mg/ml β glycerophosphate (Sigma) in 0.4 M sodium acetate buffer pH 5.0.

(ii) 30 mg/ml β glycerophosphate in 0.2 M Tris/HCl buffer pH 8.5 plus 20 mM $MgCl_2$.

(iii) 7 mg/ml 5' adenosine monophosphate (5' AMP) (Sigma) in 0.2 M Tris/HCl buffer pH 8.5 plus 20 mM $MgCl_2$.

Triplicate wells containing β glycerophosphate and 5' AMP only were included as a control for spontaneous substrate hydrolysis; triplicate wells containing cells only were used to ascertain endogenous phosphate and for protein assay.

Plates were incubated at 37°C for 2 hours and the reaction terminated by the addition of 0.4 ml of cold trichloracetic acid (20%). Plates were then spun at 1,000 rpm for 5 minutes in the plate carrying head of a Mistral (MSE) centrifuge and 0.5 ml of supernatant removed from each well and assayed for inorganic phosphate.

(ii) Inorganic phosphate assay

The assay was essentially that of Chen et al (1956). Colour reagent was prepared by dissolving 0.5 g of ascorbic acid (Sigma) in 15 ml of distilled water to which was added 5 ml of 2.5% ammonium molybdate (Sigma) and 5 ml of 3 M H_2SO_4. To 0.5 ml of colour reagent was added 0.5 ml of supernatant from wells and this mixture was incubated for 1 hour at 37°C. The colour change was read against a blank of 0.5 ml colour reagent plus 0.5 ml of distilled water in a spectrophotometer (Pye SP500) at 820 nm. Doubling dilutions of a phosphate standard from 100-0.8 µg phosphate/ml were used to construct a standard curve; total phosphate per well was calculated from the standard curve.

(iii) Protein estimation

Triplicate wells were assayed for protein content by the method of

Lowry et al (1951). Six hundred microlitres of 8 M urea were added to washed adherent cells to solubilize them and their protein content was assessed as follows. Alkaline copper reagent was prepared by adding 40 g Na_2CO_3, 1 g of sodium tartrate and 8 g of NaOH to 2 L of distilled water. Copper sulphate (0.1%) was added to alkaline copper reagent in a ratio of 1:10 to yield Lowry's first reagent. Two hundred microlitres of sample was added to 2.5 ml of Lowry first reagent and incubated for 20 minutes at room temperature. One hundred microlitres of Folin Ciocalteau reagent (BDH) were then added, mixed immediately and incubated at room temperature in the dark for 1 hour. Colour change was read in a spectrophotometer at 750 nm compared to a blank of colour reagent plus 200 µl of 8 M urea. Protein concentration of samples was obtained from a standard curve constructed from bovine serum albumin (BSA) standards (5-200 µg/ml).

(iv) Expression of results

Enzyme levels were expressed as µ moles of inorganic phosphate/mg protein/hour for nucleotidase and phosphatase. Endogenous phosphate, spontaneous substrate hydrolysis and non-specific alkaline phosphatase activity were subtracted appropriately to yield the final activity of acid phosphatase and 5' nucleotidase.

2.6 Macrophage cytotoxicity to tumour cells

The assay system used to measure the ability of macrophages to kill tumour cells was essentially the thymidine release assay of Mantovani et al (1980) with the modification of Poste (1979) whereby the counts remaining in the pre-labelled tumour cells at the end of the incubation period, were measured.

2.6.1 Tumour cell targets

Cultured CCH_1 fibrosarcoma cells, produced originally from a methylcholanthrene induced sarcoma in CBA/Ca mice and maintained in long term culture were used throughout (Woodruff et al 1972).

2.6.2 Tumour cell labelling

For use as targets in the assay CCH_1 cells were cultured in 20 ml of cRPMI with 30 μCi (2 Ci/mmol) of tritiated thymidine (Amersham International) in 75 ml flasks (Corning 25110). Maintained in this way CCH_1 cells took up label rapidly in a linear fashion until day 6 (Fig2.3) when uptake reached a plateau at approximately 2,000 cpm/10^5 CCH_1 cells in this experiment. In practice, throughout the thirty experiments carried out, the labelling varied between 1200 and 2500 cpm/ 10^5 CCH_1 cells with no obvious reason for the variation.

2.6.3 Spontaneous loss of label

Labelled CCH_1 cells maintained in culture for the duration of the assay period lost label spontaneously at a rate of approximately 10% per day (Fig. 2.4).

2.6.4 Preliminary experiments

Once CCH_1 cells were found to be reliably labelled with tritiated thymidine various methods of measuring nucleotide release were tried. A microtitre plate assay was first tried but the small number of cells used provided too few counts so 24 well plates (Falcon 3008) were used where 10^5 target cells could be used in each well. Attempts were made at first to measure the appearance of counts in the supernatant as an index of cytotoxicity but, while this was sometimes satisfactory, occasional wells had inexplicably high levels of supernatant counts which completely confounded statistical comparisons. Finally the method of Poste (1979) was adopted where the radioactivity associated with the tumour cells at the end of the assay are counted; this yielded consistently reproducible results.

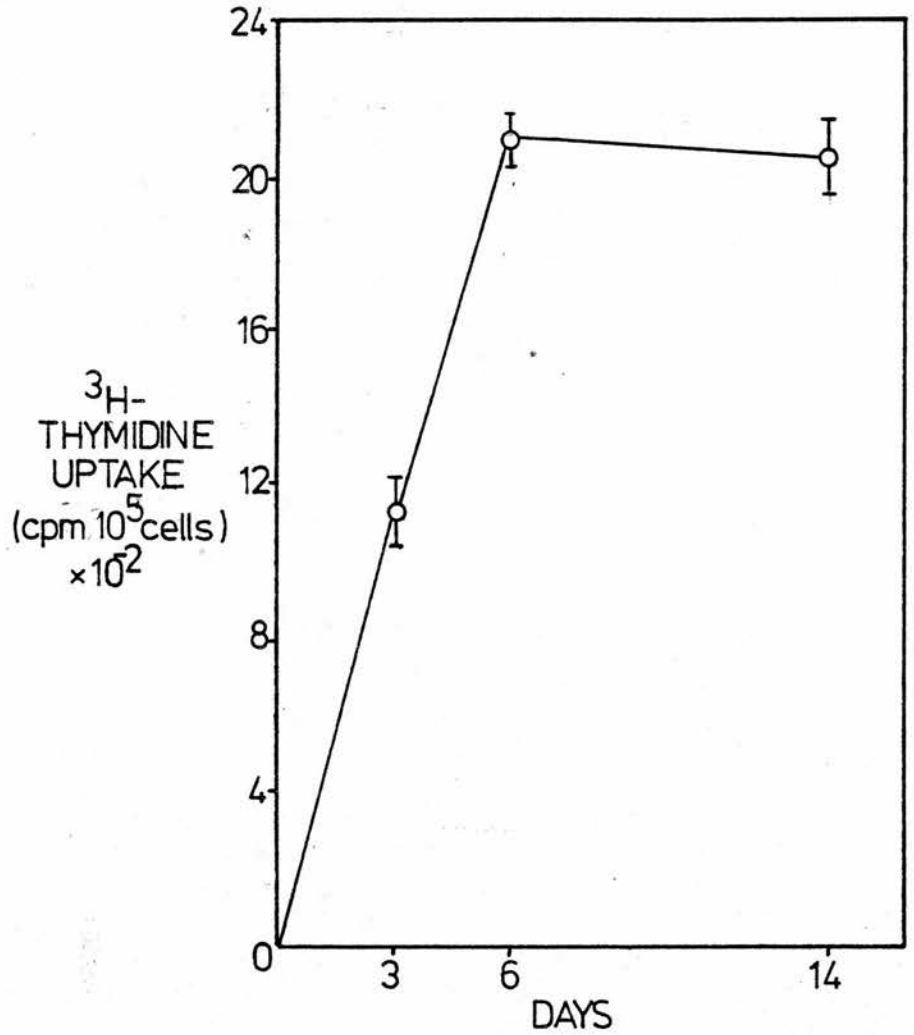


FIGURE 2.3 Uptake of ^{3}H thymidine by CCH_1 tumour cells in culture.

Symbols denote mean ± one standard deviation of triplicate wells.

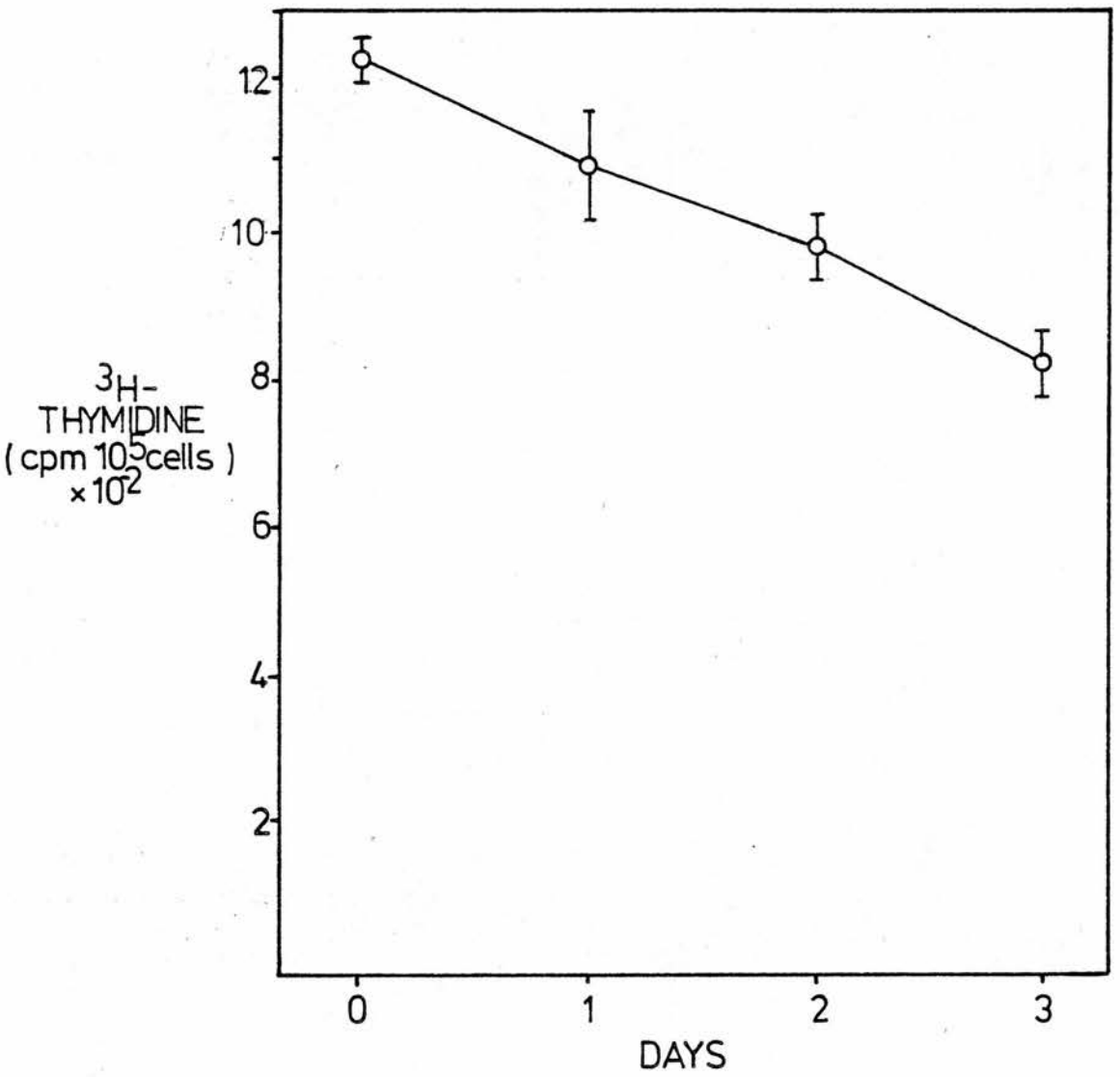


FIGURE 2.4 Spontaneous loss of label by ^{3}H thymidine labelled CCH_1 tumour cells maintained in culture for 3 days. Symbols denote mean $\pm$ one standard deviation of triplicate wells. (repres. expt.)

2.6.5 Positive control

Throughout the use of this assay, 5 day C. parvum activated macrophages were included in every experiment as positive controls since they are known to be highly cytotoxic to tumour cells in vitro.

2.6.6 Method

PEC were harvested, washed and adjusted to 10^6, $5x10^5$, 10^5 and $5x10^4$ cells/ml in cRPMI. One millilitre of each concentration was added to triplicate wells of 24 well plates and after 1 hour of incubation at 37°C in 5% CO_2, the non-adherent cells were removed by washing with Dul A. Labelled target cells, washed 3 times with Dul A and adjusted to 10^5/ml in cRPMI, were added as 1 ml to each well yielding original PEC to target ratios of 10:1, 5:1. 1:1 amd 0.5:1; 1 ml of CCM_1 cells alone was added to empty wells to determine spontaneous loss of label. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 2 days. The supernatants were then carefully removed and replaced with 1 ml of 0.2% ethylene diamine tetra-acetic acid (EDTA BDH) and left for 5 minutes at room temperature; the EDTA was pipetted up and down to further aid release of tumour cells. The 1 ml of EDTA plus tumour cells was then transferred to plastic tubes for harvesting onto glass fibre paper in a cell harvester (Skatron). The glass fibre papers with cell bound radioactivity were placed in scintillation vials (Kartell) and allowed to dry at 37°C overnight before adding 6 ml of scintillation cocktail (Burroughs). Tritium was then assessed in a β scintillation counter (Tricarb). Background counts were below 30 cpm.

2.6.7 Expression of results

Since asbestos was always injected in saline suspension and saline alone was found to induce a small amount of tumour cell cytotoxicity in

the induced macrophage population, "% specific toxicity" due to asbestos was calculated as follows, for each ratio:-

$$\% \text{ specific toxicity} = 100 \left[\frac{\text{Tumour cell bound counts following culture with asbestos induced macrophages}}{\text{Tumour cell bound counts following culture with saline induced macrophages}} \times 100 \right]$$

2.6.8 Time course of cytotoxicity

Fig. 2.5 shows the tritiated thymidine counts associated with CCM_1 cells following incubation of 10^5 labelled CCM_1 cells for up to 3 days with C. parvum and saline induced macrophages at a ratio of 10:1. It is clear that even after 1 day there is accelerated loss of label from target cells incubated with C. parvum macrophages while by 2 days (the end of a normal assay) the radioactivity remaining in targets incubated with C. parvum macrophages is only 50% of that present in tumour cells incubated with saline induced macrophages. Toxicity at day 3 was also measured and it is clear that the C. parvum toxic effect was levelling out. The counts associated with CCM_1 cells alone on day 2 show that even saline induced macrophages have some cytotoxicity against tumour cells at 10:1 ratio.

2.6.9 Validation of assay

As a validation of the assay system positively cytotoxic C. parvum macrophages plus crocidolite induced and saline induced macrophages were set up in an assay and the following two corollaries of cytotoxicity, which should accompany loss of tumour cell bound counts in the C. parvum macrophage wells, were measured

(i) reduction in the actual number of tumour cells, compared to controls, as assessed by counting in a haemocytometer.

(ii) increased appearance of counts in the supernatant (although as measured previously this was not a consistently reliable measure).

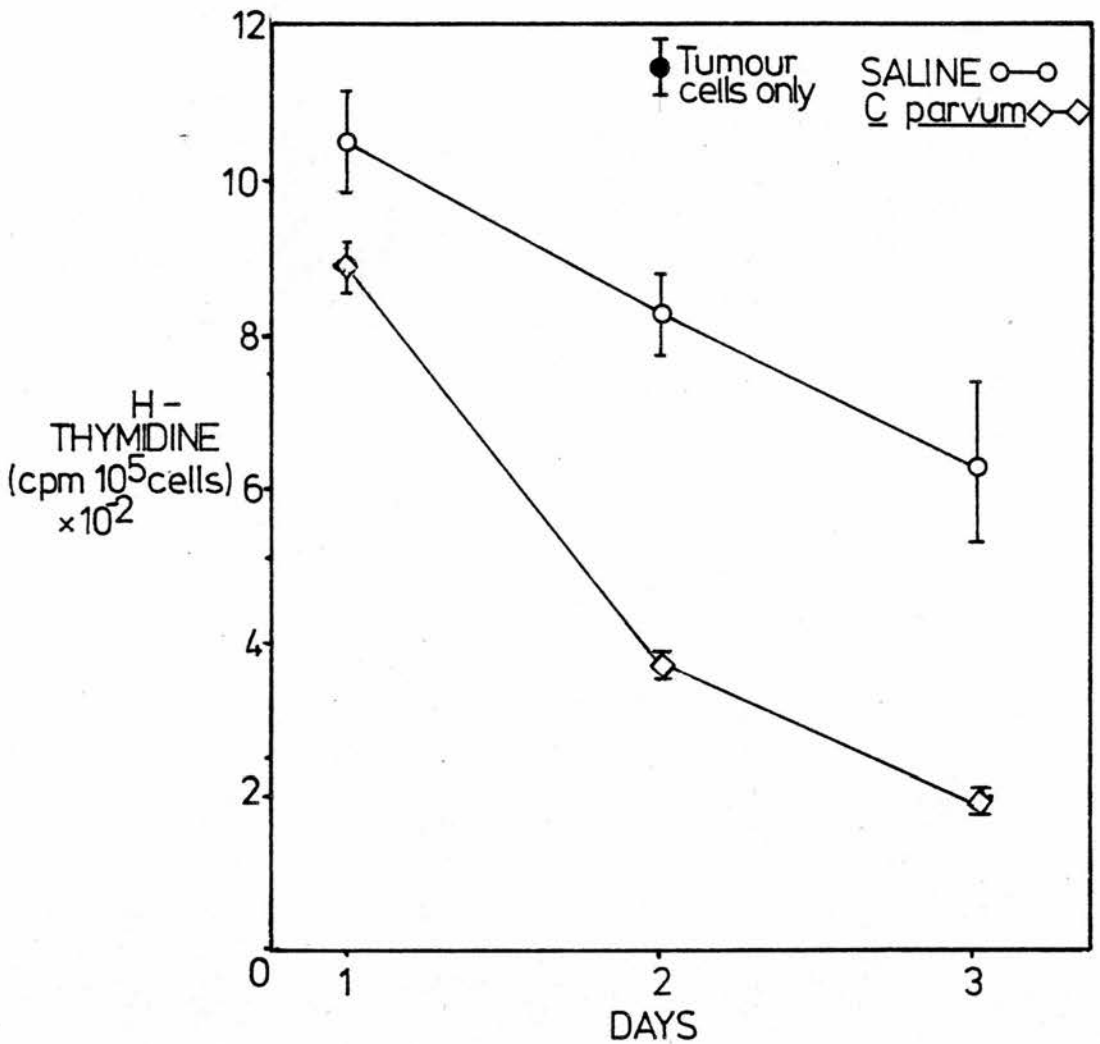


FIGURE 2.5 ^{3}H Thymidine counts in tumour cells incubated at a ratio of 10:1 with saline macrophages or C. parvum macrophages for up to 3 days.
Symbols denote $\bar{x} \pm$ one standard deviation.
triplicate wells (repr. exp^t)

Fig.2.6 shows that both of these conditions were satisfied and that, accompanying the decrease in tumour cell bound counts in the C. parvum macrophage wells, there was a decrease in the number of tumour cells and an increase in the supernatant counts, compared to the other two conditions.

2.6.10 Elimination of tritiated thymidine uptake by macrophages as a potential confusing factor

Other experiments in this study have shown that macrophages can take up tritiated thymidine in considerable amounts and this, allied to the likelihood that some macrophages would be released by EDTA, necessitated that this potentially confusing factor in the assay be checked. In order to check this the maximum number of macrophages (i.e. derived from 10^6 3 day saline, 5 day C. parvum, 3 day crocidolite and chrysotile PEC) were plated into wells, washed and incubated in 2,500 cpm tritiated thymidine; this dose represented more than the maximum of counts which could be released by a 50% kill of well labelled CCM_1 cells. These macrophage cultures were then EDTA treated and harvested in the usual manner. Table 2.1 shows the results of the experiment showing that only background counts were harvested. Uptake of tritiated thymidine by macrophages and their possible release by EDTA amongst the tumour cells was therefore ruled out.

2.6.11 Elimination of the possible confusing effect of tumour cells not being killed but merely floating off

In order to check that viable tumour cells were not being discarded with the supernatant before EDTA was added at harvesting, the following was carried out. A typical C. parvum macrophage kill was set up including a dose response and the supernatants were retained, at the end of the incubation period and, instead of being discarded, they were harvested for cell bound counts. As Fig. 2.7 shows there was a normal dose

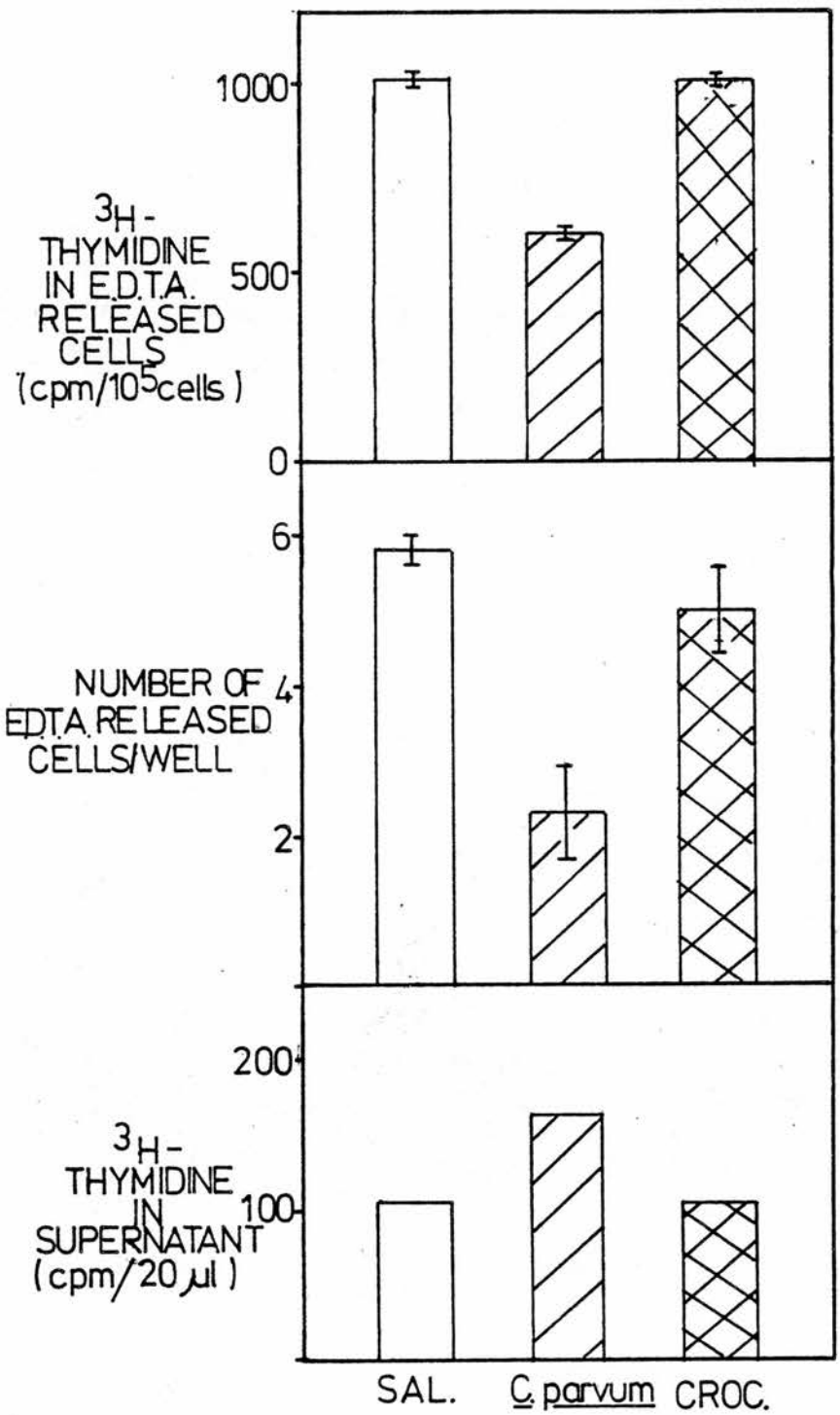


FIGURE 2.6 Figure demonstrating that reduction of counts in EDTA released tumour cells (top) following incubation with C. parvum is correlated with a decrease in the number of tumour cells which can be counted visually (middle) and an increase in the supernatant counts (bottom).
Symbols denote $\bar{x} \pm$ one standard deviation.

TABLE 2.1 Harvestable counts obtained from macrophages (derived from 10^6 PEC) induced by various agents and incubated with 2,500 cpm tritiated thymidine for 2 days.

Macrophage Source	Harvestable Counts
3 day saline	25 ± 14
3 day crocidolite	17 ± 9
3 day chrysotile	17 ± 10
5 day *C. parvum*	19 ± 6

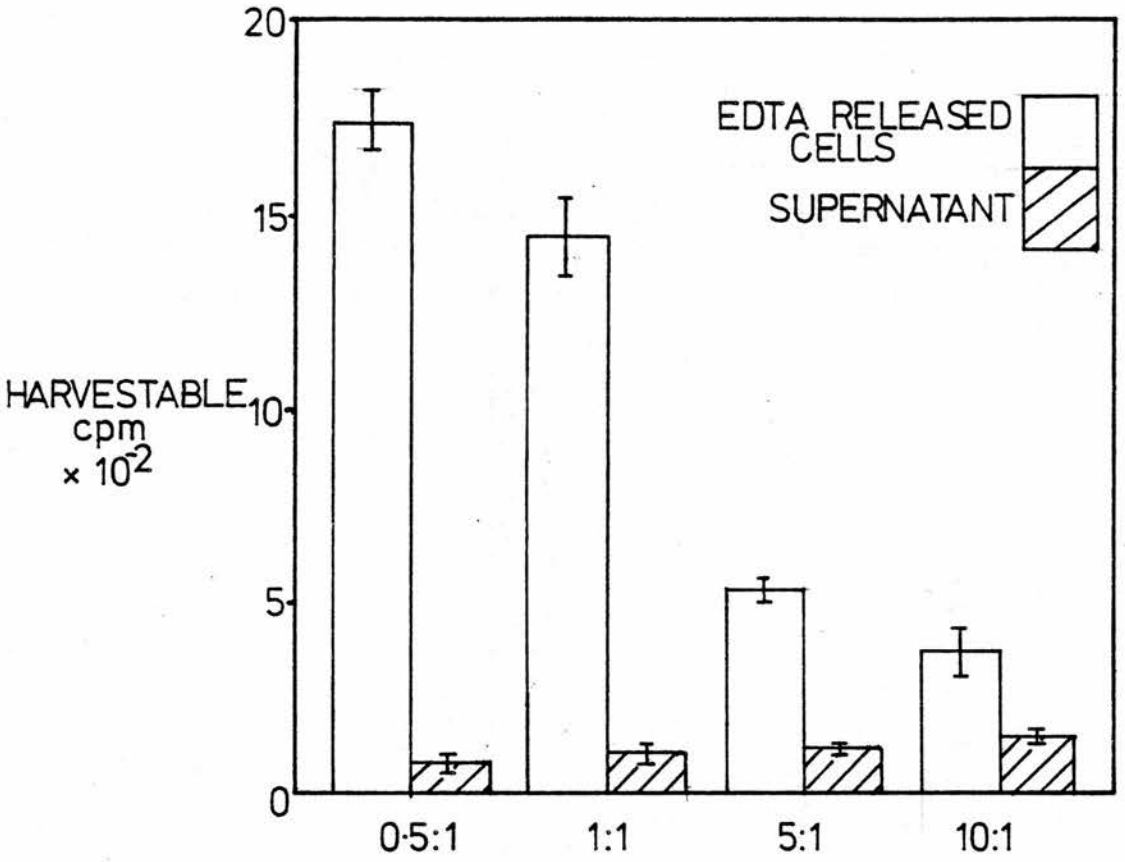


FIGURE 2.7 **Harvestable EDTA released cell bound counts and supernatant counts at various effector target ratios with C. parvum induced macrophages.**

$\bar{x} \pm sd$ of triplicate wells (repr.ve exp.)

response in the killing as measured by decreased counts in the EDTA released tumour cells while the harvestable counts in the supernatant remained at the same low level throughout the dose response. The absence of harvestable counts in the supernatant showed that "floating off" of labelled tumour cells did not contribute to the loss of tumour cell bound counts during a C. parvum activated macrophage mediated tumour cell kill.

2.7 Assay for the effect of macrophage supernatants, asbestos and asbestos leached medium on CCM$_1$ tumour cell proliferation

2.7.1 Macrophage supernatants

PEC harvested 3 days after ip saline, chrysotile or crocidolite, or 5 days after ip C. parvum were cultured in 75 ml flasks (Corning 25110) (6.0×10^6/flask) in cRPMI for 1 hour then washed to remove non-adherent cells. The macrophages remaining were then incubated for a further 24 hours in 15 ml cRPMI after which the supernatants were harvested, centrifuged, aliquoted and frozen at -20°C until use.

2.7.2 Asbestos

Crocidolite and chrysotile were prepared at 5 mg/ml in cRPMI and incubated at 37°C for 1 hour then diluted in cRPMI to yield 0.01, 0.1, 1, 10 and 100 µg/100 µl.

2.7.3 Leachate(medium in which asbestos has been incubated)

Crocidolite and chrysotile asbestos at 5 mg/ml in cRPMI were incubated for 3 days at 37°C with daily mixing. The suspension was then spun at 3,500 g for 50 minutes and the asbestos free supernatant Millipore filtered (0.2 µm) to sterility.

2.7.4 Preliminary experiments

Since it had been decided to utilise a microtitre plate assay,

experiments were undertaken to decide the best inoculating dose of CCH_1 cells to use in each well. Various concentrationf of CCH_1 cells were prepared such that 100 µl yielded 10^4, $2x10^4$, $5x10^4$, 10^5 and 10^6 cells; after 24 hours 0.25 µCi of tritiated thymidine were added to each well; wells were harvested after a further 24 hours. Uptake of tritiated thymidine (measured as in 2.6.6) was seen to peak in cultures containing $5x10^4$ - 10^5 CCH_1 cells (Fig.2.8) so $5x10^4$ was routinely used in subsequent assays.

2.7.5 Method

CCH_1 cells were harvested from culture, washed and adjusted to $5x10^5$/ml; 100 µl of this suspension were added to the wells of microtitre plates (Sterilin M29 ARTL). Depending on the agent whose effect was sought the following were then added to triplicate wells:-

(i) macrophage supernatants at 5, 25 and 50% of the total volume (200 µl).

(ii) asbestos at 0.01, 0.1, 1, 10 or 100 µg in 100 µl of cRPMI.

(iii) asbestos leachate as 5, 25 and 50% of the total volume.

Plates were incubated at 37°C in 5% CO_2 for 24 hours at which point 0.25 µCi of tritiated thymidine were added to each well; wells were harvested after a further 24 hours and the cell bound tritium assessed as described previously(2.6.6). Microscopic examination of wells before and after harvesting revealed that virtually all tumour cells were harvested from wells.

2.8 In vivo assays to measure the effect of asbestos on growth of subcutaneous CCH_1 tumours

2.8.1 Subcutaneous tumour growth

CCH_1 tumour cells were harvested from culture, washed 3 times with Dul A and adjusted in Dul A so that 0.1 ml gave the required inoculating

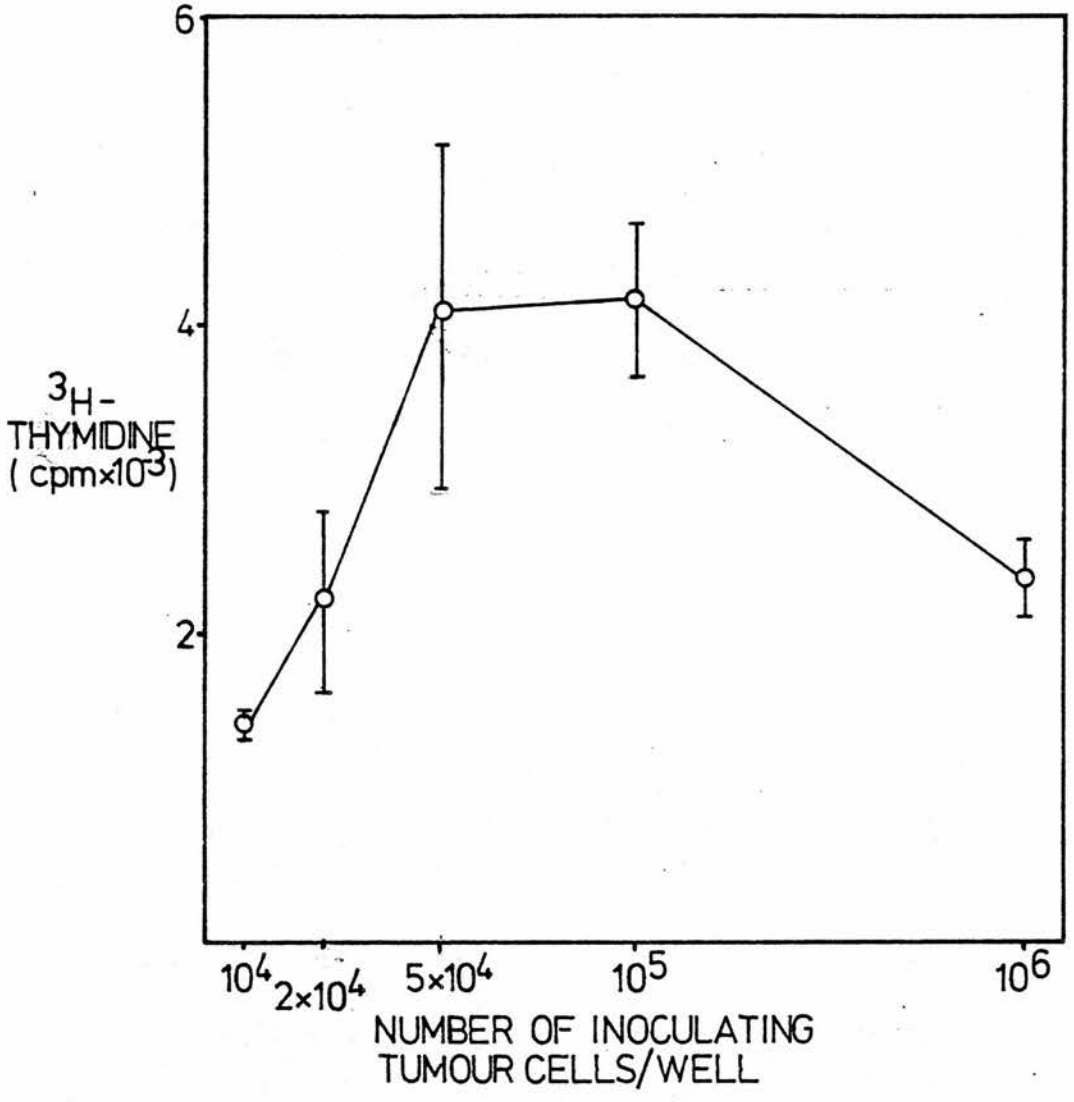


FIGURE 2.8 **Effect of number of inoculating CCH_1 tumour cells on subsequent uptake of ^{3}H thymidine.**
$\bar{x}\pm sd$ of triplicate wells (repr.^{ve} exp^t.)

dose of 10^6 or $5x10^2$ cells. Mice were prepared in groups of 10 and received 0.1 ml of tumour cell suspension subcutaneously in the exterior lateral aspect of the right hind leg; in this position the growth of the tumour could be monitored using a caliper calibrated to 0.5 mm.

2.8.2 Asbestos injection

Asbestos was injected ip within 3 days before or after tumour cell inoculation. Crocidolite or chrysotile asbestos was used at 2.5, 5 or 20 mg in 0.5 ml Dul A; Dul A alone and 0.2 ml (1.4 mg) of C. parvum were also injected as controls.

2.8.3 Winn type assays

In this assay PEC induced by asbestos or saline were mixed at 10:1 or 100:1 with CCH_1 cells and injected subcutaneously in 0.1 ml volume containing 10^5 CCH_1 cells plus 10^6 PEC (10:1) or $5x10^2$ CCH_1 cells plus $5x10^4$ PEC (100:1); controls consisting of the appropriate number of CCH_1 cells alone were also used.

2.8.4 Effect on tumour growth of mixing crocidolite with the inoculating tumour cells

CCH_1 cells were harvested, adjusted to the required concentration and divided into 4 aliquots. To separate aliquots were added 5, 50, 500 or 0 µg crocidolite as a constant volume (10 µl) of cRPMI. The crocidolite had been pre-incubated with cRPMI for 1 hour at 37°C before dilution. Mice were injected subcutaneously with 0.1 ml containing 500 CCH_1 cells plus 0, 5, 50 or 500 µg of crocidolite. The growth of the tumours was monitored.

2.8.5 Effect on tumour growth of having grown the inoculating tumour cells in vitro in the presence of crocidolite

2.5 µg of crocidolite asbestos was pre-incubated in cRPMI for 1 hour

then added to a culture flask newly seeded with $0.5x10^6$ CCH_1 cells which were then incubated for 4 days. CCH_1 cells were then harvested, adjusted to 500 CCH_1/0.1 ml and 0.1 ml injected subcutaneously into 10 mice; 10 mice received the same dose of CCH_1 cells grown in parallel, but untreated, culture. Tumour growth was monitored.

2.9 Use of Concanavalin A to study the effect of asbestos on macrophage membranes

2.9.1 Visualisation of Concanavalin A receptors in macrophages during interaction with asbestos in vitro - light microscopy

Concanavalin A (Con A) conjugated to fluorescein isothiocyanate (FITC-Con A) (Sigma) was dissolved in Dul A at 20 µg/ml. Control macrophage cultures were prepared as previously indicated (2.5.2) and 100 µl of chrysotile asbestos (50 µg/ml) in RPMI (with or without 10% foetal calf serum) was added to each coverslip. Cultures were then incubated at 37°C in 5% CO_2 for 10, 30 or 60 minutes to allow for chrysotile/macrophage membrane interaction. Coverslips were then washed in Dul A and incubated in FITC-Con A for 20 minutes at room temperature after which they were washed in Dul A, fixed in ethanol for 5 minutes and mounted in Dul A/Glycerol 1:1. Coverslips were viewed and photographed in an American Optical Company, Phase Star light microscope with an ultraviolet light source and photographic attachment. All photographs were exposed on Tri X Pan film (Kodak).

2.9.2 Electron microscopy

The demonstration of Con A binding sites by the peroxidase method was taken from Garrido et al (1974). Control macrophage cultures were prepared in plastic petri-dishes (50 mm Sterilin 122) and chrysotile asbestos (50 µg/ml) was added in RPMI with or without 10% foetal calf serum; plates were incubated for 5, 10, 30 or 60 minutes. Only

chrysotile could be used since this is the only asbestos type which is readily cut by ultramicrotomy.

At appropriate time points monolayers were washed and fixed by the addition of glutaraldehyde in cacodylate buffer (see section 2.5.6) for 5 minutes. Cultures were then washed a further 3 times and incubated with Con A (Taab laboratories) (50 µg/ml Dul A) for 15 minutes at 4°C, after which time the cultures were washed 3 times and incubated with peroxidase (Taab laboratories) (50 µg/ml Dul A) for 15 minutes at room temperature. The substrate for the demonstration of peroxidase was prepared immediately before use by adding 1 drop of 30% H_2O_2 (Taab laboratories) per 5 ml of 3.3 diaminobenzidine (Taab laboratories) (0.5 mg/ml in 0.1 M Tris buffer pH 7.4). This substrate was added to the culture plates and incubated for 15 minutes in the dark at room temperature. Cultures were then washed 3 times with PBS and fixed in OsO_4 fixative (see 2.5.6). The cells were then harvested with a rubber policeman and processed to araldite. Unstained sections were viewed in the electron microscope where Con A/peroxidase sites were evident as black reaction product at the cell surface. Control cells processed for endogenous peroxidase showed very occasional pale intracellular staining with no cell membrane staining.

2.9.3 Con A induced capping of macrophages

PEC induced by various stimuli were set up as coverslip cultures, allowed to adhere and the non-adherent cells removed by washing. Coverslips were then placed on racks and 200 µl of FITC-Con A (Sigma) (20 µg/ml Dul A) were added and the coverslips incubated for 20 minutes at 37°C; occasionally shorter or longer periods of incubation were used. Coverslips were then washed with PBS, fixed in ethanol for 5 minutes and mounted in Dul A/glycerol 1:1. The coverslips were finally examined

in a UV microscope and the percentage of capping macrophages in 200 cells was determined for each coverslip.

(i) Specificity of FITC-Con A binding To check the specificity of FITC-Con A binding to the macrophage surface, macrophages were:-

(a) pre-incubated in non-fluoresceinated Con A followed by FITC-Con A treatment for 20 minutes.

(b) treated with FITC-Con A in the presence of 50 mM methyl mannoside.

With both of these treatments membrane fluorescence of macrophages was abolished and pinocytic uptake of FITC-Con A was seen to be minimal.

(ii) Temperature requirement for capping The percentage of capping macrophages was determined for C. parvum and chrysotile activated macrophages treated with FITC-Con A at room temperature or 37°C for 20 minutes.

(iii) Time course of capping C. parvum and chrysotile activated macrophages were incubated at 37°C for various times and the percentage of capping cells determined.

(iv) Pharmacological modification of capping In experiments to assess the role of microtubules and microfilaments in Con A induced capping of activated macrophages the following pre-treatments were given:-

1. colchicine (Sigma) 10^{-4}M.
2. cytochalasin B (Sigma) 10^{-4}M.
3. lignocaine hydrochloride (Phoenix Pharmaceuticals) 10^{-2}M

all given as 100 µl of a Dul A solution for 1 hour at 37°C followed by incubation with FITC-Con A for 20 minutes at 37°C; percentage of caps in 200 randomly counted cells was assessed. Since cytochalasin B was dissolved initially in dimethyl sulphoxide (DMSO) (BDH), a suitable dilution of DMSO was also used to pretreat macrophages to preclude any

effect of DMSO alone on capping.

(v) Dose response of capping in chrysotile induced macrophages Mice were injected with 0.005, 0.05, 0.125, 0.5, 1.25 and 2.5 mg of chrysotile asbestos in 0.5 ml Dul A, and the percentage of capping macrophages in the 3 day induced macrophages ascertained.

(vi) Re-expression of Con A receptors after capping Three day saline and chrysotile induced macrophage cultures were treated with non-fluoresceinated Con A (20 µg/ml) at 37°C for 20 minutes then incubated in cRPMI for up to 4 hours to allow re-expression of Con A receptor sites. At 0, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours and 4 hours the macrophage cultures were treated with FITC-Con A at 37°C for 20 minutes and then scored for brightness of fluorescence and proportion of caps.

2.9.4 Assessment of number of macrophage membrane Con A binding sites by $[^{125}I]$ Con A binding

(i) Iodination of Con A The method used was essentially that of Hunter (1978). Con A (Pharmacia) and Na $[^{125}I]$ (Amersham International)were used. One ml of Con A (10 mg/ml Dul A) was placed in an ice cooled bijou containing a small magnetic stirrer; 100 µl of 0.5 M phosphate buffer pH7.5 and 10 µl of Na $[^{125}I]$ (14.1 mCi/µg iodine: 100 mCi/ml) were then added. To start the iodination reaction 100 µl of chloramine T (BDH) (20 mg/ml Dul A) were added and the reaction allowed to continue for 30 seconds. To stop the reaction 100 µl of sodium metabisulphate (40 mg/ml Dul A) were added. The stopped reaction mixture was passed down a Sephadex G25 column which had been pre-coated with 300 µl of Bovine Serum Albumin (Pentex) (20% W/V) and equilibrated with PBS; 10x3 ml fractions were eluted with PBS into glass tubes. Ten microlitre samples from each fraction were placed in 5 ml glass tubes and counted in a gamma counter (LKB); 100 µl of 5% foetal calf serum was then added to each tube followed by 4 ml of 20% trichloracetic acid (TCA) and each tube was capped and vortexed. The TCA precipitate was sedimented by centrifugation, the supernatant discarded and the TCA precipitable counts assessed.

A graph showing the results of a typical iodination is shown as Fig. 2.9 The TCA precipitable counts were 94% of the total counts in the 4th and 5th fractions which were pooled for use in assays. The $[^{125}I]$ Con A, all of which was assumed to have been eluted, was made up to 10 ml (1 mg/ml) aliquoted and frozen until use.

(ii) Stability of $[^{125}I]$ Con A A batch of $[^{125}I]$ Con A was assessed for stability one month after iodination and, as shown in Table 2.2 dissociation was found to be less than 5% per month. $[^{125}I]$ Con A was only kept for 2 months maximum.

(iii) Choice of an assay system for measurement of $[^{125}I]$ Con A binding to macrophages Several assay systems were considered for measuring the binding of $[^{125}I]$ Con A to macrophage surfaces. The methods used were essentially the same as for the coverslip method eventually adopted. Normal PEC ($5x10^5$) were added to glass or plastic tubes pre-treated, or not, with 5% BSA (Pentex) for 1 hour at 37°C. In the case of the microtitre plate assay $5x10^4$ PEC were added to each well. An arbitrary 1/10 dilution of $[^{125}I]$ Con A was used. After washing to remove non-adherents, macrophages were treated with $[^{125}I]$ Con A for 15 minutes at room temperature then washed extensively. The counts in tubes and control tubes with no cells were assessed in a gamma counter. In the case of the microtitre plate assay the washed wells were separated with a hot wire before gamma counting. In the case of the sodium dodecyl sulphate (SDS) technique the test tubes had the $[^{125}I]$ Con A labelled cells solubilized in 2 ml of SDS (20%) for 10 minutes with mixing; the counts in the SDS solubilized macrophages and SDS washes of $[^{125}I]$ Con A treated control tubes were then assessed.

Fig. 2.10 shows the amount of non-specific binding, due to adsorption of the $[^{125}I]$ Con A to the surfaces of the tubes and wells tried. It is clear that microtitre plates, plastic and glass tubes uncoated and coated

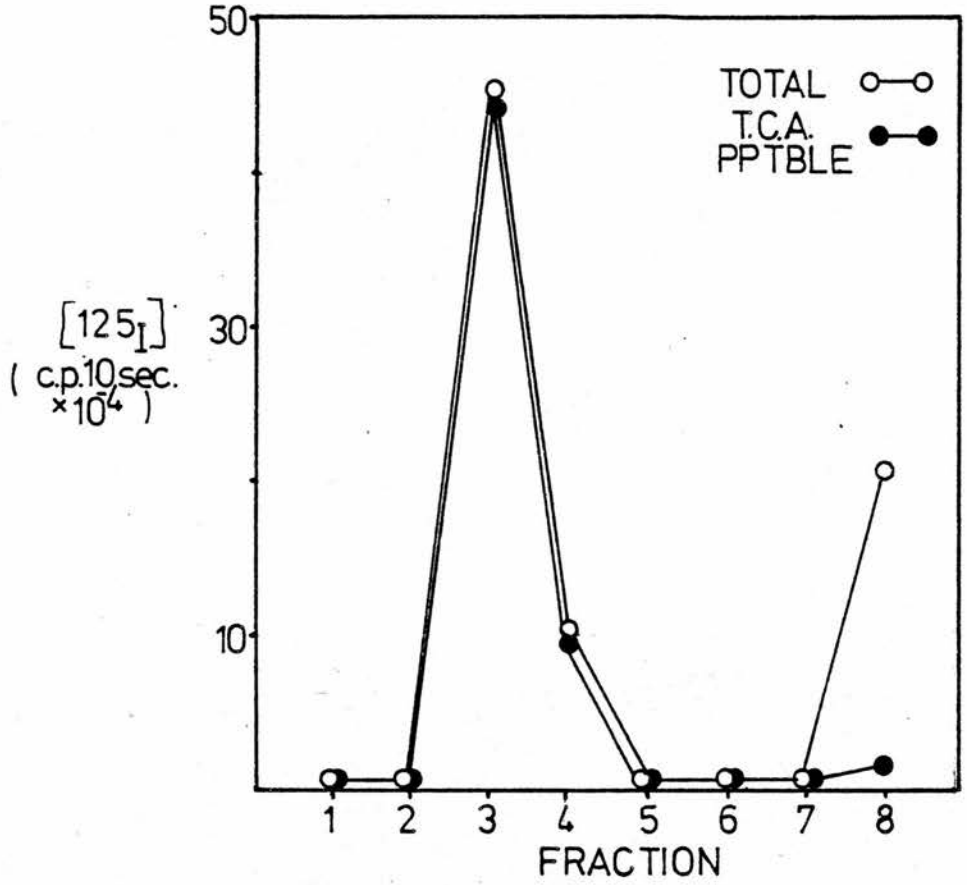


FIGURE 2.9 Result of a typical Con A iodination.

TABLE 2.2 Change in percentage of TCA precipitable [^{125}I] Con A with time.

Date	Total Counts	TCA Precipitable Counts	%
10/12/80	452,321	424,360	93.8
8/1/81	201,549	181,376	89.9

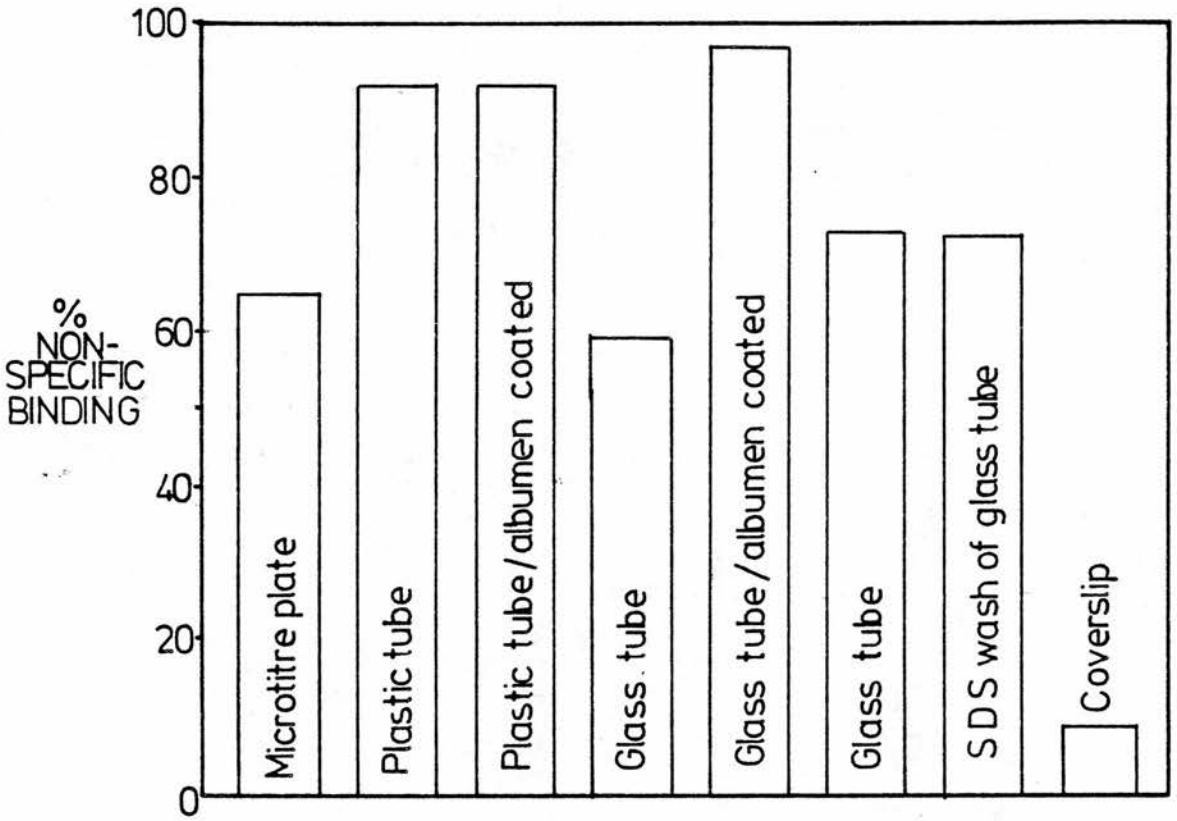


FIGURE 2.10 Non-specific binding in various $[^{125}I]$ Con A binding assay systems tried.

with BSA and SDS solubilization all resulted in very high background counts. The glass coverslip method therefore proved to be the best system with regard to non-specific binding and was adopted for use.

(iv) Coverslip method of measuring $[^{125}I]$ Con A binding to macrophages

The method used was essentially the method of Lutton (1973). In preliminary experiments PEC were collected, washed and the percentage of adherent cells was assessed for 3 day saline, 3 day chrysotile and 5 day C. parvum PEC. This was done by plating 10^6 PEC onto plastic petri-dishes (50 mm Sterilin 122) in cRPMI and incubating for 1 hour at 37°C in 5% CO_2; the non-adherents were then removed with 3 consecutive Dul A washes and the washes kept and pooled. The pooled washes were centrifuged, brought into a small volume and counted in a haemocytometer to yield the percentage non-adherents. The percentage of macrophages (100 - % non-adherents) was:- saline 74.2%; chrysotile 49.3%; C. parvum 65.6%. The PEC were adjusted to yield 4.5×10^6 adherent cells/ml in each case and 100 µl of suspension were pipetted on to 6x22mm coverslips (Chance-Propper). The coverslips had been washed in Decon and extensively rinsed in tap and distilled water. Coverslips on racks were incubated for 1 hour at 37°C in 5% CO_2 to allow for macrophage adherence then washed vigorously in PBS to remove non-adherents. To each coverslip was added 100 µl of $[^{125}I]$ Con A and the coverslips incubated for 15 minutes at room temperature. The $[^{125}I]$ Con A was drained off and the coverslips washed, using forceps, in 5 large volumes of PBS before placing in plastic tubes and assessing the bound $[^{125}I]$ counts.

(v) Concentration of Con A Binding to control macrophages (4.5×10^5) was found to have plateaued by 200 µg/ml (Fig. 2.11) so this concentration of $[^{125}I]$ Con A was used routinely.

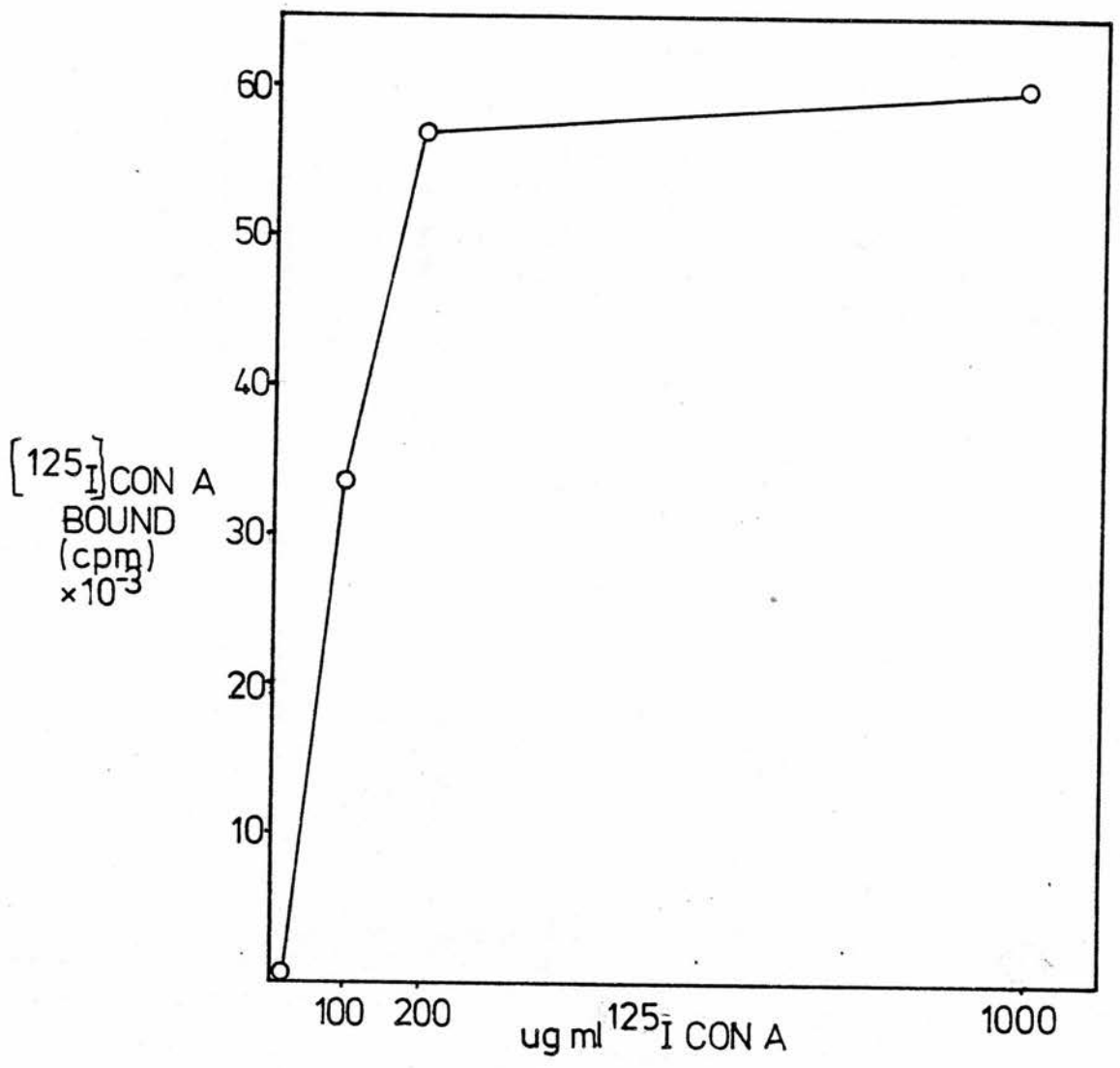


FIGURE 2.11 **Effect of $[^{125}I]$ Con A concentration on binding to control macrophages.**

$\bar{x}$ of duplicate coverslips

(vi) Number of target macrophages Increasing the number of target macrophages/coverslip caused a linear increase in the amount of binding (Fig. 2.12).

(vii) Time course of $[^{125}I]$ Con A binding Binding of $[^{125}I]$ Con A to macrophages was found to have reached a plateau by 15 minutes at room temperature (Fig. 2.13).

(viii) Specificity of $[^{125}I]$ Con A binding In order to test the specificity of $[^{125}I]$ Con A binding the binding was carried out in the presence of 50 mM methyl mannoside; binding was reduced to background by this procedure (Table 2.3).

(ix) Elimination of endocytosis of $[^{125}I]$ Con A as a potential confusing factor To test whether endocytic uptake of $[^{125}I]$ Con A could contribute significantly to binding levels, the binding of $[^{125}I]$ Con A was carried out in the presence of 2 inhibitors of endocytosis - 2 mM sodium azide and 1 mM sodium iodoacetate. No significant effect on binding was produced by inhibiting endocytosis (Table 2.4).

(x) Latex phagocytosis To measure the effect of latex phagocytosis on $[^{125}I]$ Con A binding, control macrophage cultures were prepared and 100 µl of 1% latex (Bacto latex 0.81 µm diameter, Difco) in cRPMI were added and incubated for 1 hour at 37°C in 5% CO_2. $[^{125}I]$ Con A binding to latex treated macrophages and control macrophages was then carried out as normal.

(xi) Asbestos phagocytosis In some experiments control macrophages were incubated for 1 hour at 37°C with 100 µl of chrysotile (50 µg/ml) in cRPMI and $[^{125}I]$ Con A binding assessed.

(xii) Antibody coated SRBC phagocytosis Antibody coated SRBC's (Ab SRBC's) were prepared with 1:50 dilution of anti-SRBC serum (as given in 2.5.5) and 100 µl were added to control macrophage cultures which were incubated at 37°C for 1 hour. Control and Ab SRBC treated cultures were then treated with 100 µl of red blood cell lytic buffer for 2 minutes before

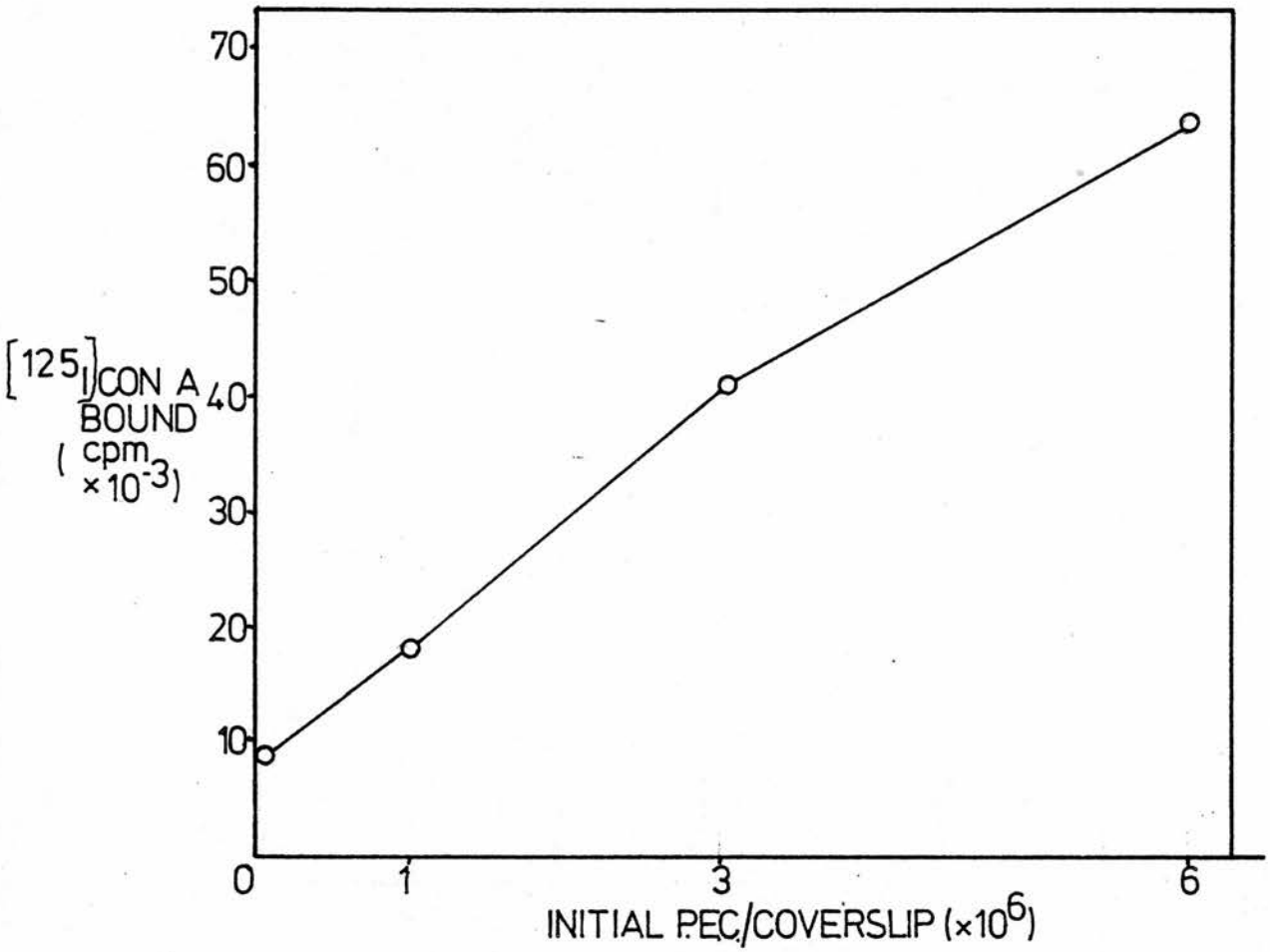


FIGURE 2.12 **Effect on $[^{125}I]$ Con A binding of increasing the number of target cells.**

$\bar{x}$ of duplicate c/slips

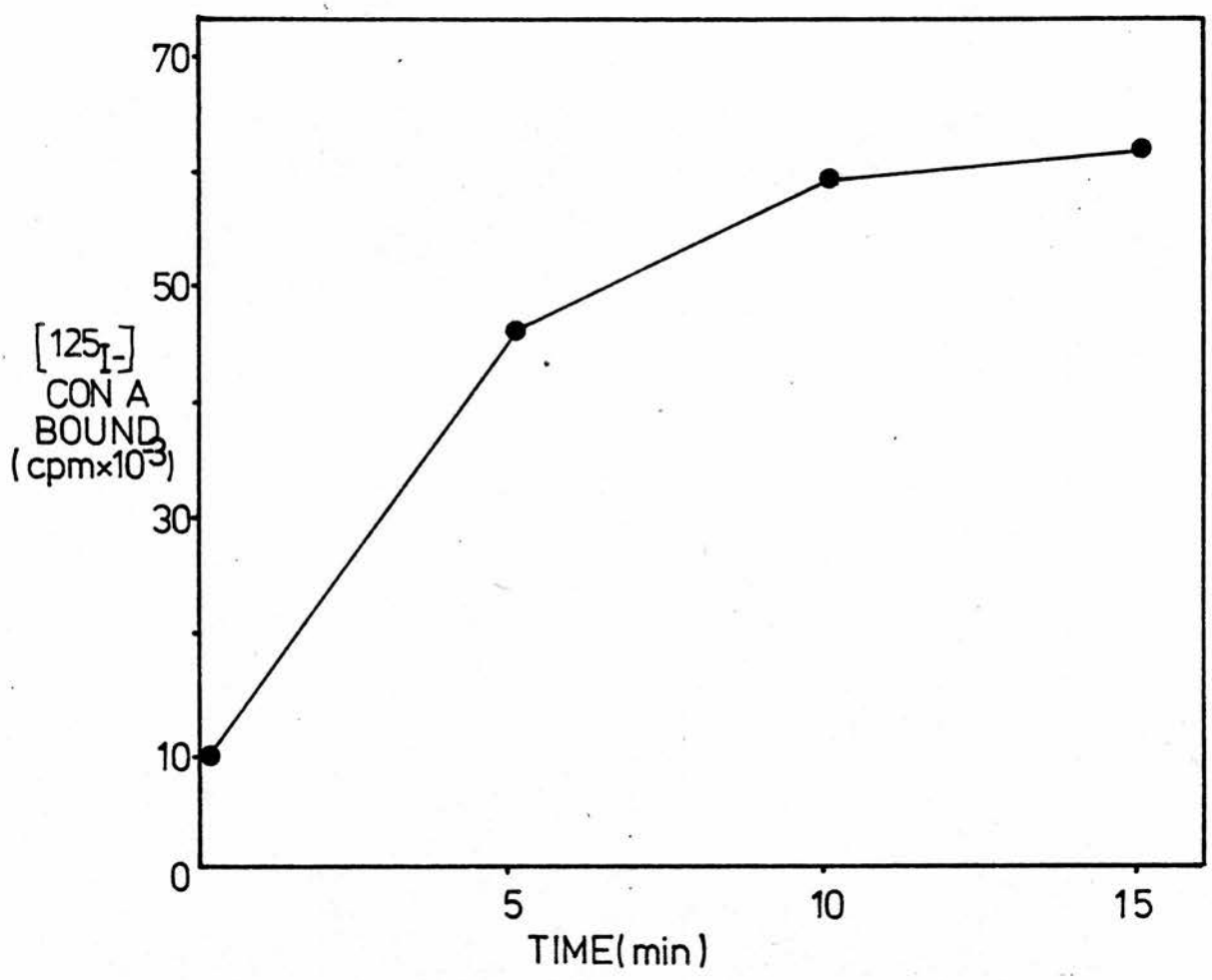


FIGURE 2.13 **Time course of $[^{125}I]$ Con A binding to macrophages.**
$\bar{x}$ of duplicate c/slips

TABLE 2.3 Effect of carrying out $[^{125}I]$ Con A binding in the presence of 50 mM methyl mannoside.

Condition	$[^{125}I]$ Con A Bound (cp 30 seconds)
Blank coverslip	5,367 ± 234
Macrophages + $[^{125}I]$ Con A	41,326 ± 10,073
Macrophages + $[^{125}I]$ Con A + Methyl mannoside	4,742 ± 707

*

TABLE 2.4 Effect of the endocytic inhibitors iodoacetate and azide on $[^{125}I]$ Con A binding.

Treatment	$[^{125}I]$ Con A Bound (cp 30 seconds)
Saline	67,957 ± 10,074
Iodoacetate	64,356 ± 5,352
Azide	56,863 ± 12,727

Blank 8,357 ± 1051

*

* $\bar{x} \pm sd$ of 3 separate exp^s

washing in 3 volumes of PBS. $[^{125}I]$ Con A binding was then measured.

(xiii) Trypsin releasability of bound $[^{125}I]$ Con A A normal $[^{125}I]$ Con A binding experiment was carried out and the counts released by enzyme treatment assessed according to the method of Marques (1978). Coverslips were placed in tubes containing 2 ml trypsin (1 mg/ml Dul A) (Difco) or, in initial experiments in pronase (2.5 mg/ml Dul A) (Calbiochem Behring). Coverslips were left in enzyme for 15 minutes at room temperature, agitated in enzyme solution with forceps then transferred to another tube. Enzyme released and coverslip retained counts were then determined; enzyme released counts were expressed as a percentage of total counts.

2.10 Effects of asbestos induced macrophage supernatants on lymphocyte mitogenesis

2.10.1 Thymocyte mitogenesis assay

Thymuses were obtained under sterile conditions from 6 week old CBA/Ca mice and gently disaggregated in a glass homogenizer in Dul A. The large debris was allowed to settle and the supernatant containing free thymocytes was decanted; the thymocytes were washed 3 times with Dul A and adjusted to $5x10^6$/ml in cRPMI supplemented with $5x10^{-5}$M 2 mercapto-ethanol. Aliquots of cell suspension were adjusted to 0, 2, 4 and 5 µg/ml Concanavalin A (Pharmacia) by adding a small volume of concentrated Con A in Dul A. One humdred microlitres of cell suspension were added to the wells of microtitre plates (Sterilin M29 ARTL); 100 µl of test medium, e.g. macrophage supernatant, were then added to triplicate wells. This produced a final concentration of 0, 1, 2 and 4 µg/ml Con A in 200 µl volume. The plates were then incubated at 37°C in 5% CO_2 for 48 hours at which point 0.25 µCi of tritiated thymidine were added to each well. After a further 24 hours the wells were harvested in a cell harvester and the cell bound tritiated thymidine determined as described

previously (2.6.6) by scintillometry. In some preliminary experiments label was added after 72 hours and the wells harvested at 96 hours.

A typical dose response curve for Con A mitogenesis of thymocytes is shown in Fig.2.14 and it is clear that there is an optimal dose above which tritiated thymidine uptake is inhibited.

2.10.2 Attempts to obtain Interleukin 1 (IL1) activity in supernatants of macrophages activated in vitro

Normal PEC were seeded into 75 ml flasks (Corning 25110) at $6x10^6$/ flask in cRPMI and the non-adherents removed after 1 hour. The medium was replaced with cRPMI containing 50 µg/ml bacterial lipopolysaccharide (LPS)(Difco). Flasks were incubated at 37°C in 5% CO_2 for 24 or 72 hours after which the supernatants were collected, centrifuged, aliquoted and stored at -20°C.

2.10.3 Attempts to obtain IL1 activity in supernatants of macrophages activated in vivo

Mice were injected with 0.5 ml proteose peptone (Difco) (10%) or 0.5 ml Con A (Pharmacia) (500 µg/0.5 ml) and left for 3 days after which time the PEC were harvested and the adherent cells incubated in serum-containing or serum-free RPMI for 24 or 48 hours. The supernatants were harvested, centrifuged and dialysed overnight against RPMI at 4°C. Supernatants were then filtered to sterility and made up to cRPMI where appropriate.

2.10.4 Preparation of supernatants with suppressor activity, from asbestos induced macrophages

Twenty-four hour supernatants were prepared in cRPMI for 3 day crocidolite, 3 day chrysotile, 3 day latex , 3 day saline, 10 month crocidolite and 10 month saline induced macrophages. Supernatants were obtained as described above.

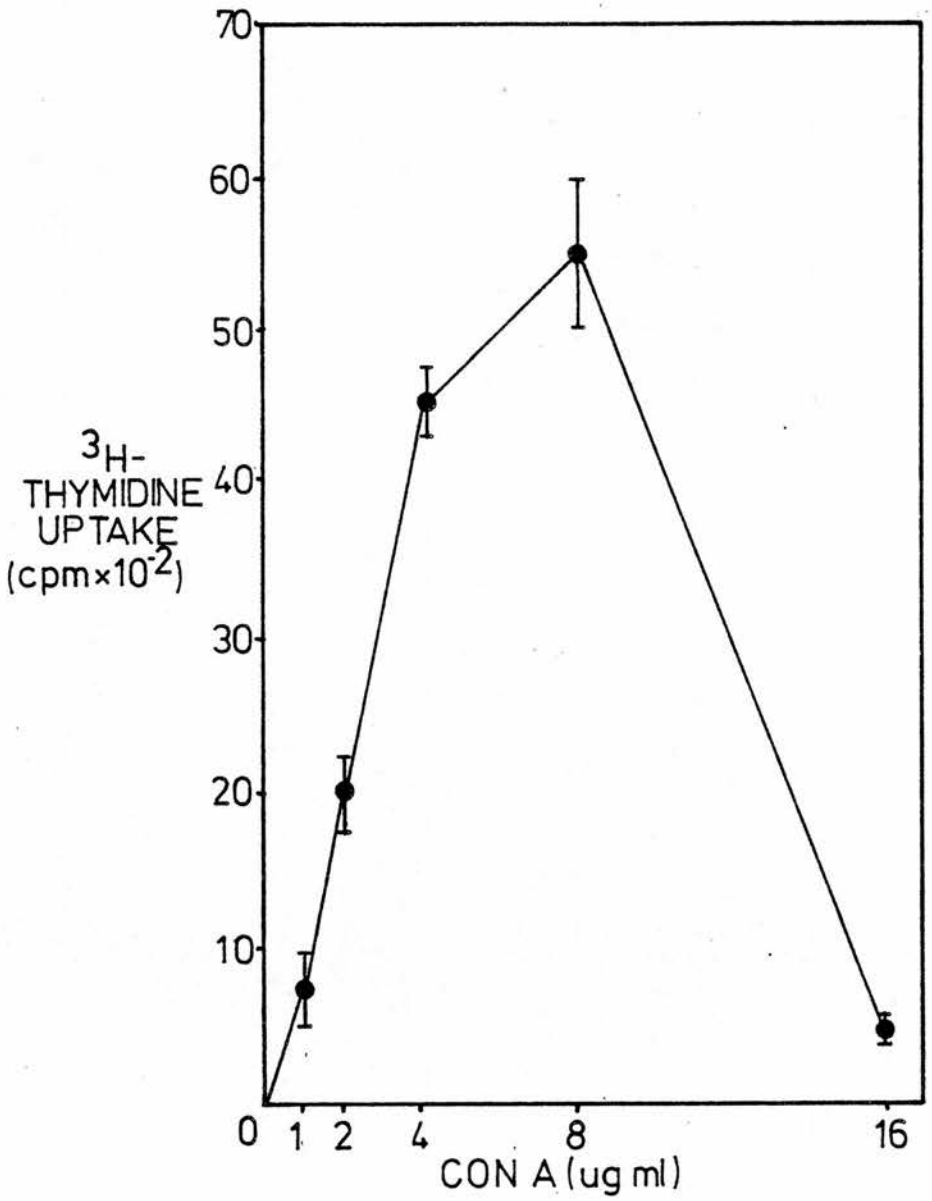


FIGURE 2.14 **Effect of Con A concentration on thymocyte mitogenesis as measured by ^{3}H thymidine uptake.**

$\bar{x}$±sd. of triplicate wells (repr.ve exp.)

2.10.5 Supernatants from macrophages treated with chrysotile in vitro

Adherents obtained from $5x10^6$ control PEC were incubated in 75 ml flasks in cRPMI containing 500 µg chrysotile/ml. At the end of 72 hours of incubation more than 70% of macrophages were dead as assessed by dye exclusion. The 72 hour supernatant was centrifuged at 3,500 G for 30 minutes, aliquoted and stored at -20°C.

2.10.6 Dialysis

Three day chrysotile macrophage supernatant was dialysed overnight against RPMI at 4°C.

2.10.7 Serum

Serum collected from 3 day saline, 3 day crocidolite and 3 day chrysotile injected mice was incorporated in the thymocyte mitogenesis assay at 1 and 10%.

2.10.8 Asbestos

Asbestos was preincubated for 1 hour at 37°C in cRPMI then diluted so that 100 µl contained 0.001, 0.01, 0.1, 1 or 10 µg; 100 µl of asbestos solution were incorporated into wells in thymocyte mitogenesis assays.

2.10.9 Effect of supernatants on fibroblast proliferation

An early passage mouse kidney fibroblast cell line kindly provided by Dr. Jan Boyd was used as a target to test the effect of supernatants on non-lymphoid cells. The proliferation assay used was essentially the same as that used for tumour cells (2.7.5) except that the target cells were incubated for 10 minutes in 0.5 per cent trypsin before harvesting.

2.10.10 Preparation of splenic lymphocytes

CBA/Ca mouse spleen cells were obtained under sterile conditions and gently dispersed in a glass homogeniser. Approximately 10^8 mononuclear

cells were obtained from one spleen. The cells obtained from one spleen were suspended in 0.5 ml of cRPMI and applied to a nylon wool column (Leukopac) prepared in a 10 ml plastic syringe (Sterilin) according to the method given in Mishell and Shiigi (1980) and equilibrated with cRPMI at 37°C for 1 hour. The splenocytes were layered on to the top of the nylon wool and allowed to enter the column; a further 2 ml of cRPMI were added and the column closed. The column was incubated at 37°C for 1 hour after which time the non-adherent cells were eluted with 15 ml of cRPMI.

The cells eluted from the column, and those which could be obtained by mechanical agitation of the nylon wool after elution of the non-adherents, were characterized by surface immunofluorescent staining with anti-θ (prepared in the Department of Surgery) to detect T lymphocytes and anti-Ig (Dako) to detect B lymphocytes. As shown in Table 2.5 the column eluted cells were 80% T lymphocytes and contaminated with only 2% of B lymphocytes; the remaining cells were presumed to be macrophages. Similarly the cells which could be obtained by mechanical agitation of the nylon wool, which would be predicted to be B lymphocytes, were 80% surface Ig positive and contained only 16% θ positive cells.

2.11 Attempts to detect differences in surface antigenicity between saline and asbestos induced macrophages

2.11.1 Raising of anti-sera

Mature New Zealand White (NZW) rabbits bred in the Department of Surgery were used. On day 0 PEC was collected from mice injected ip 6 days previously with 0.5 ml Dul A or 2.5 mg crocidolite in 0.5 ml Dul A. The PEC were collected in RPMI 1640 containing 10 U/ml heparin (Pularin) and no serum. PEC were washed 4 times with Dul A and adjusted

TABLE 2.5 Surface staining characteristics of (a) splenocytes eluted from a nylon wool column and (b) cells obtained by mechanical agitation of the wool after (a).

	Percentage	
	+	-
Anti θ	80	20
Anti Ig	2	98

(a)

	+	-
Anti θ	16	84
Anti Ig	80	20

(b)

to $5x10^7$ in 0.2 ml Dul A. The PEC were then added to 1.5 ml of Complete Freunds Adjuvant (Difco), placed in a laboratory blender and mixed for 5 minutes until a drop of the mixture remained stable on a water surface. This adjuvant mixture was injected subcutaneously into the left flank. Rabbit 151 received the saline PEC; rabbit 152 received the crocidolite PEC.

This procedure was repeated on day 14 substituting Incomplete Freunds Adjuvant (Difco), and injected subcutaneously into the right flank.

On day 28 $5x10^7$ washed PEC in 0.2 ml Dul A were injected into the ear vein of the appropriate rabbits.

2.11.2 Harvesting and storage of anti-sera

Rabbits were pre-bled from the ear vein on day -2 and were bled from the ear vein to obtain anti-sera on days 42 and 56; on day 70 the animals were exsanguinated. All blood samples were kept overnight at $4^{\circ}C$ in universal containers to allow clotting, then spun at 3,500 G for 10 minutes. The serum was separated from the clot and spun once more to remove all blood cells. Sera was then complement de-activated by incubating in a water bath at $56^{\circ}C$ for 30 minutes, aliquoted and stored at $-20^{\circ}C$ until use.

2.11.3 Assay of antibody binding to macrophages

The iodination of Protein A and subsequent assay were carried out essentially as described by Brown et al (1979).

2.11.4 Iodination of Protein A

A bijou, in a beaker of ice, was set up on a small magnetic mixer and 30 µl of Protein A (1 mg/ml PBS) (Pharmacia) were added. To this were added 100 µl 0.5 M phosphate buffer (pH 7.2), 10 µl Na ^{125}I (14.1 mCi/µg iodine : 10 mCi/ml) (Amersham International) and 10 µl of Chloramine T

(1 mg/ml PBS) (Sigma); the solution was allowed to mix on ice for 20 minutes. To terminate the reaction 20 µl of sodium metabisulphite (1 mg/ml PBS) and 20 µl of sodium iodide (20 mg/ml) were added. The stopped reaction mixture was passed down a 25 cm Sephadex G25 column pre-coated with 200 µl BSA(50 mg/ml) and equilibrated with PBS; 2.5 ml fractions were eluted with PBS into glass tubes and the TCA precipitable counts assessed as described earlier (2.9.4). In the fractions pooled to obtain the working reagent there were approximately 90% TCA precipitable counts. Fig. 2.15 shows the result of a typical Protein A iodination.

2.11.5 $[^{125}I]$ Protein A binding assay

Whole PEC PEC were harvested in the usual manner 6 days after ip injection of 0.5 ml saline or 2.5 mg crodicolite in 0.5 ml saline. PEC were washed 3 times and adjusted to $5x10^5$/ml in diluent (5% FCS in PBS). Two hundred microlitres of PEC suspension were added to triplicate wells (Remov-a-well system M74A Dynatech) in a polystyrene "plate". As with all other centrifugations in this assay the plates were then centrifuged in the plate carrying head of an MSE Mistral centrifuge at 1,000 rpm for 5 minutes. The cells were then resuspended by holding against a whirlimixer and 200 µl of anti-serum or prebleed serum, suitably diluted, were added. The plates were incubated on ice for 1 hour. Plates were then spun and washed 3 times before adding 100 µl of $[^{125}I]$ Protein A (10^5 cpm/100 µl) and leaving to incubate on ice for 45 minutes. After this incubation 100 µl of diluent were added and the cells spun and washed a further 4 times. Wells were then allowed to dry overnight before counting in a gamma-counter.

The following controls were always included:-

(i) replacing the anti-serum step with diluent to measure non-specific Protein A adherence.

(ii) prebleed serum was used to check for Fc mediated attachment of

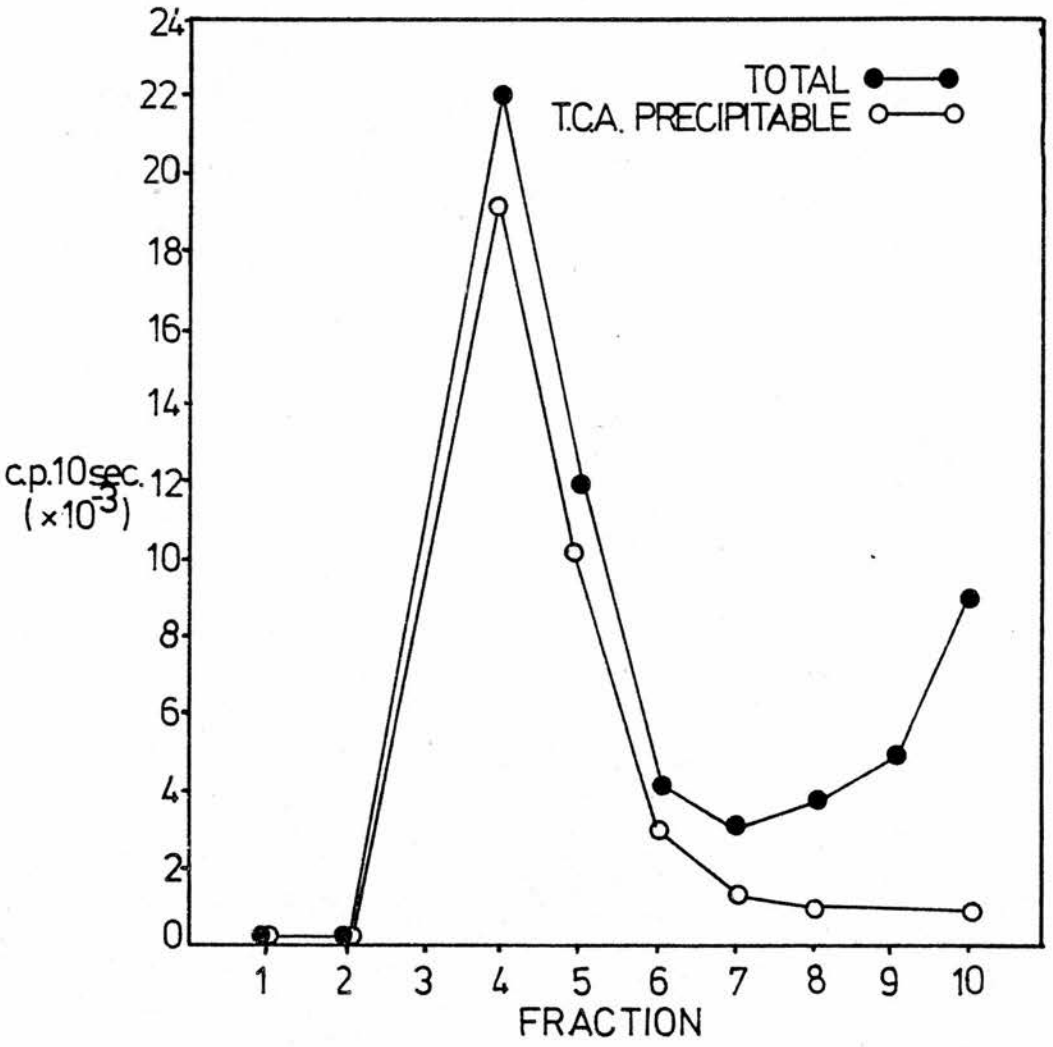


FIGURE 2.15 Result of a typical Concanavalin A iodination showing that the vast majority of $[^{125}I]$ in fractions 4 and 5 are protein bound.

antibody and cross reactive antibody which could be present in normal serum.

Adherent cells In later experiments only adherent cells were used and allowed to adhere for 1 hour before washing to remove non-adherents. The assay was otherwise the same as for whole PEC except that centrifugation was unnecessary.

2.11.6 Absorption of anti-sera

Absorptions were carried out by mixing 100 µl of anti-serum with up to 10^8 CBA/Ca thymocytes and incubating on ice for 1 hour.

2.11.7 Immunofluorescence

(i) Anti-sera Absorbed anti-serum to 6 day saline or 6 day crocidolite PEC was used.

(ii) Developing anti-globulin Fluorescein isothiocyanate conjugated goat anti-rabbit IgG (FITC-G∝R) (Seward) was used at a dilution of 1/30 in Dul A to detect binding of anti-serum.

(iii) Method Coverslip cultures of 6 day saline or 6 day crocidolite macrophages were prepared, washed and 100 µl of absorbed anti-serum (1/10 dilution in Dul A) were placed on them for 30 minutes at room temperature. Coverslips were then washed and uncubated with 100 µl of FITC-G∝R at room temperature for 30 minutes. Coverslips were then washed 3 times with PBS and mounted on slides in PBS:glycerol (1:1) and examined in a UV microscope.

(iv) Blocking experiments In experiments designed to detect differences between anti-sera, with regard to binding to macrophages, coverslip cultures were treated with a first anti-serum as above followed by unlabelled G∝R; macrophages were then treated with the second anti-serum followed by FITC-G∝R. In this way only sites not recognised by the first anti-serum but recognised by the second were

revealed. The number of macrophages showing "ring" fluorescence in 200 cells was expressed as a percentage for each treatment.

2.11.8 Complement mediated lysis

Anti-sera absorbed with 10^8 thymocytes /100 µl were prepared at 1:10 dilution in 5% BSA in Dul A and guinea pig serum, as a source of complement, was added as 25% V/V. One hundred microlitres of anti-serum and appropriate controls were added to coverslip cultures of 6 day saline and 6 day crocidolite induced macrophages and incubated at 37°C for 2 hours. Coverslips were then washed, 100 µl of 0.05% Nigrosin added and left at room temperature for 10 minutes before inverting on a glass slide and counting the percentage of dead cells in 200 macrophages. The following controls were used in the incubation step:-

(a) Dul A/BSA

(b) anti-sera plus 25% heat inactivated (30 minutes at 56°C) guinea pig serum.

(c) Dul A/BSA plus 25% guinea pig serum

(d) prebleed serum plus 25% guinea pig serum

(e) to test that the guinea pig serum was working 1/25 dilution of horse anti-thymocyte serum was used against thymocytes.

2.12 Use of indirect $[^{125}I]$ Protein A binding to measure autoimmune deposition of antibody on the surfaces of asbestos induced macrophages in vivo

Attempts were made to detect the presence of immunoglobulin on the surface of asbestos activated macrophages using an indirect $[^{125}I]$ Protein A binding assay.

2.12.1 $[^{125}I]$ Protein A binding assay

PEC were obtained 6 days, 14 days and up to 11 months after ip injection of 2.5 mg crocidolite, chrysotile or Dul A and adjusted to

$2x10^6$ PEC/ml in cRPMI. One hundred microlitres of cell suspension was delivered into Remov-a-wells (Dynatech M74 A) in a well holding plate and spun for 5 minutes at 1,000 rpm as described above. Plates were then incubated for 3 hours at 37^0C in 5% CO_2 to allow for macrophage adherence. The non-adherent cells were removed with 3 washes with 200 µl of cold diluent (Dul A containing 5% FCS). Two hundred microlitres of various dilutions of rabbit anti-mouse immunoglobulin antiserum (Dako) in diluent were added to triplicate wells and left on ice for 1 hour. Wells were then washed, incubated with $[^{125}I]$ Protein A, washed and the radioactivity associated with each well ascertained as described for the indirect Protein A assay (2.11.5).

Controls

(a) Dul A/FCS was substituted for anti-serum to measure non-specific $[^{125}I]$ Protein A adherence.

(b) To demonstrate that $[^{125}I]$ Protein A was working, anti-sera to PEC (151/2 unabsorbed) was used at 1/50 dilution.

(c) To demonstrate that rabbit anti-serum to mouse Ig was working and detecting mouse Ig, spleen cells which had been treated with a monoclonal mouse anti θ (1/50 dilution 1 hour at 0^oC) then washed, were included.

2.13 Long term effects of intraperitoneal asbestos

A number of mice were injected with asbestos and kept for up to 2 years to assess the validity of the CBA/Ca mouse peritoneal cavity as a model in comparison with the known pathological effects of asbestos.

2.13.1 Histopathology

When mice showed signs of ill health or, in some cases, at predetermined times, they were killed by ether overdose and the peritoneal viscera resected and fixed in Bouin's solution. Tissue was processed

to wax then cut and stained with haematoxylin and eosin. Other tissues including tumours were treated in the same way.

2.13.2 Attempts to passage ascitic tumours by direct transfer

To determine whether the ascites which developed in many long term asbestos injected mice, was malignant, ascites was removed by syringe under sterile conditions from freshly killed mice. One millilitre was injected directly into recipient syngeneic mice which were monitored for ascites development.

2.13.3 Cell lines from ascites

On two occasions ascites from long term asbestos injected mice was brought into culture as $5x10^6$ cells/flask (75 ml Corning 25110) and maintained in primary culture for up to 6 weeks in cRPMI. If piled up colonies developed, the flasks were treated with Trypsin/EDTA (Gibco) for 5 minutes at 37°C and the cells so obtained were subcultured until a cell line was established.

2.13.4 Assay of tumourigenicity of cell lines

Subcutaneous site Cells were removed from culture, washed and adjusted to 10^6 cells/0.1 ml Dul A and 0.1 ml injected subcutaneously into the right hind limb of mice. Animals were observed for tumour growth.

Intraperitoneal site Cells were obtained as above and adjusted to 10^6 cells/0.5 ml Dul A; 0.5 ml was then injected ip and the mice observed for tumour development.

Tumours from the subcutaneous sites were occasionally disaggregated with enzymes by incubating diced tumour with Trypsin (0.1 mg/ml) (Difco), Pronase (0.25 mg/ml) (Calbiochem Behring), DN ase (0.1 mg/ml) (Calbiochem Behring) at 37°C for 30 minutes with occasional shaking. Freed cells were cultured as given above.

2.13.5 Limited characterization of 13 MC1 Asc cell line

This cell line was chosen to carry out some preliminary characterization due to its ability to produce mesothelioma-like tumours on ip injection.

(a) Immunogenicity 13 MC1 Asc cells were irradiated with 22,000 RADS and mice received 10^6 irradiated tumour cells ip on day 0. On day 14 they received 10^6, 10^5, 10^4 or 10^3 normal 13 MC1 Asc ip. Other mice received no prior immunisation but received 10^6, 10^5, 10^4 or 10^3 13 MC1 Asc. Mice were monitored for tumour development.

(b) pI4 antigen expression Attempts to detect the "pI4" marker of the transformed phenotype (Bowen and Kulatilake 1979), were carried out using the $[^{125}I]$ Protein A assay for immunoglobulin binding as described above (2.11.5). Rabbit anti-serum to "pI4" was a gift from Dr. D. Mannant.

2.14 Statistical analysis

Mean and standard deviation of replicates were calculated on pocket calculators (TI Programmable 58 Texas Instruments and Cassio fx 3600P), and the means compared using Student's T test (Bailey 1959).

RESULTS

3.1 Validity of the CBA/Ca mouse peritoneal model

A major factor in justifying the use of the mouse peritoneal injection model to study the biological effects of asbestos is that it reacts with lesions analogous to those produced in the lung by asbestos ; these pulmonary responses have been described extensively in Chapter 1 and are fibrogenesis and carcinogenesis. Mice were therefore injected intraperitoneally with asbestos or control solutions and kept long term to check the development of pathology. Further details are given in Section 3.8.

3.1.1 Fibrogenic response

All mice killed more than 10 weeks after asbestos injection had some degree of fibrosis in the peritoneal cavity and this was evident at autopsy as binding together of the peritoneal viscera making separation of the organs very difficult. At light microscopy the reason for this was seen to be extensive fibrous adhesions binding the gut and visceral organs together (Fig. 3.1); these fibrotic masses often contained visible asbestos fibres.

3.1.2 Carcinogenic response

Many mice injected with asbestos and kept for more than a year developed ascites and when this was transferred intraperitoneally to syngeneic mice, tumours were produced in a small proportion of cases. When the primary ascites was cultured, piled up colonies of cells arose which, on injection, formed fibrosarcomas at subcutaneous sites and mesothelioma-like tumours on injection into the peritoneal cavity (Fig. 3.2).

In these studies UICC asbestos injected into the peritoneal cavity of CBA/Ca mice elicited a fibrogenic and carcinogenic response while saline, latex and C. parvum controls did not.

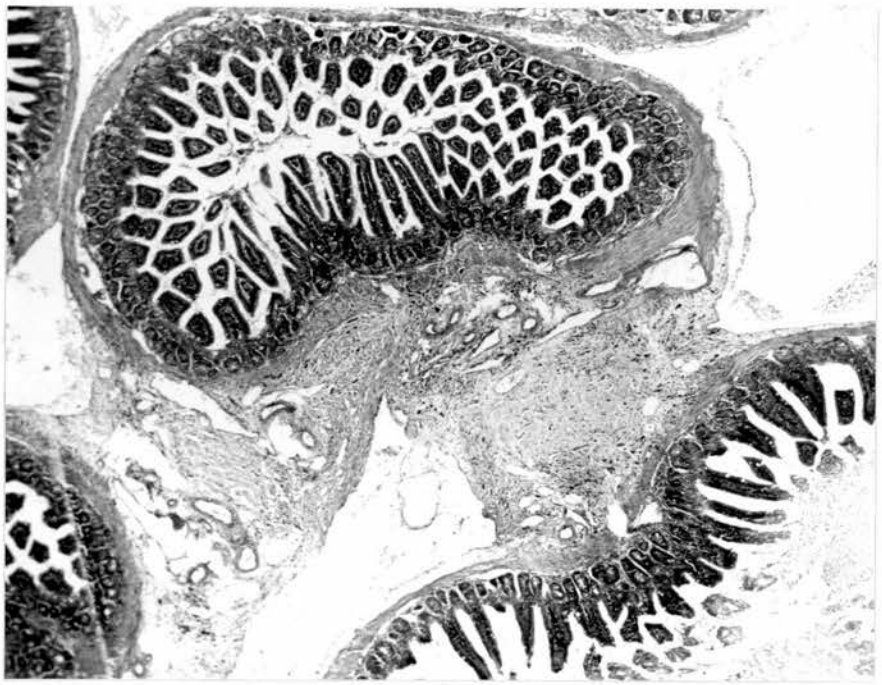


FIGURE 3.1 Section of the peritoneal viscera of a mouse injected with crocidolite 13 months previously. Large fibrous masses are visible binding sections of gut together

Mag. x 60

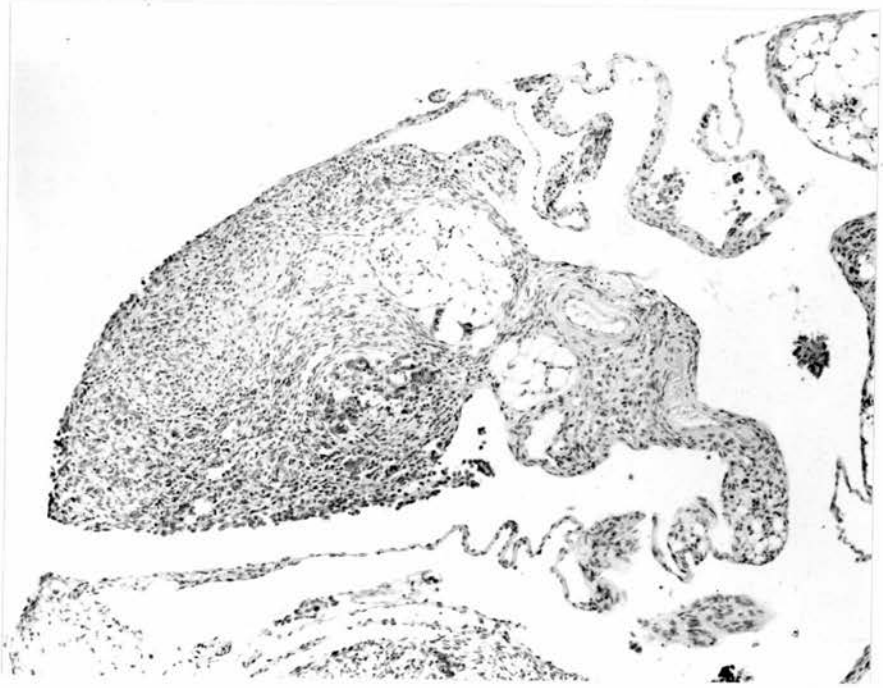


FIGURE 3.2 Nodular tumour arising in the peritoneal cavity following intraperitoneal injection of a cell line (13MC1) derived from ascites which developed in mouse injected with crocidolite

Mag. x 150

3.2 Effects of intra-peritoneal asbestos on some properties of the induced macrophage population in the short, medium and long term

3.2.1 Number of PEC Fig. 3.3 shows the total number of PEC harvested from mice at the 3 time points, 3, 18 and 70 days after injection of saline, latex, crocidolite or chrysotile. The number of PEC induced by asbestos was significantly ($P < 0.05$ to $P < 0.002$ see Fig. 3.3 for details) greater than that induced by saline on days 3 and 70; there was no significant difference between saline or asbestos PEC numbers on day 18. The number of PEC induced by latex injection was never significantly greater than that induced by saline.

The exact cellular composition of the PEC was not ascertained at each time point but macrophages averaged between 50 and 70% of the cells with lymphocytes and neutrophils comprising the remainder; neutrophils were particularly increased in proportion in the 3 day asbestos induced populations.

3.2.2 Macrophage phagocytosis

Fig. 3.4 shows that on day 3 both asbestos types had induced macrophages with a lesser mean proportion of cells able to phagocytose latex particles than saline induced macrophages. Only crocidolite induced macrophages however were significantly ($P < 0.05$) less able to phagocytose than saline induced macrophages. At the two subsequent time points there was no significant difference with regard to phagocytic ability.

3.2.3 Fc rosettes

Fig. 3.5 shows the percentage of rosette forming macrophages at the 3 time points. The total rosette forming cells were detected with 1/10 dilution of sensitizing antiserum and the high affinity receptor bearing cells with a 1/1000 dilution of sensitizing antiserum. The mean total percentage of rosette forming cells was only once below 90% with all

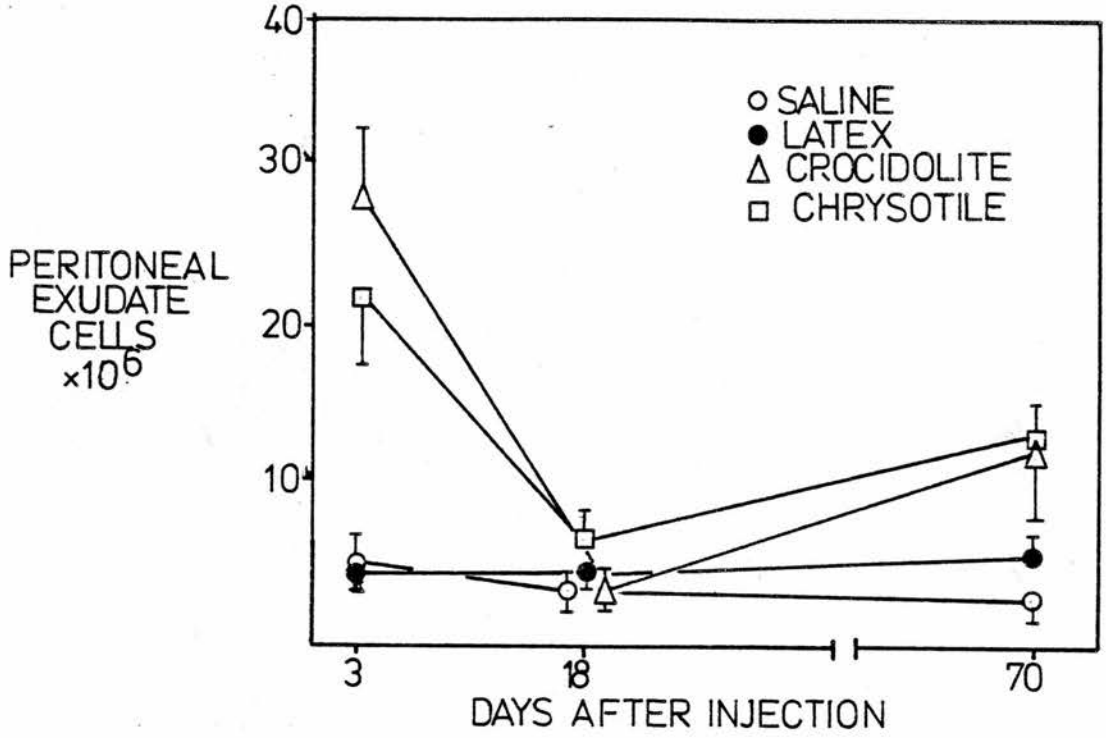


FIGURE 3.3 Mean number of peritoneal exudate cells harvested from mice 3, 18 or 70 days after injection of 0.5 ml Dul A 2.5 mg crocidolite, 2.5 mg chrysotile or 0.1% latex. Significant differences between asbestos and saline:- 3 days crocidolite $P<0.002$, chrysotile $P<0.01$; 70 days crocidolite $P<0.05$, chrysotile $P<0.01$; latex was never significantly different from saline.
Bars denote $\pm$ one standard deviation.
*

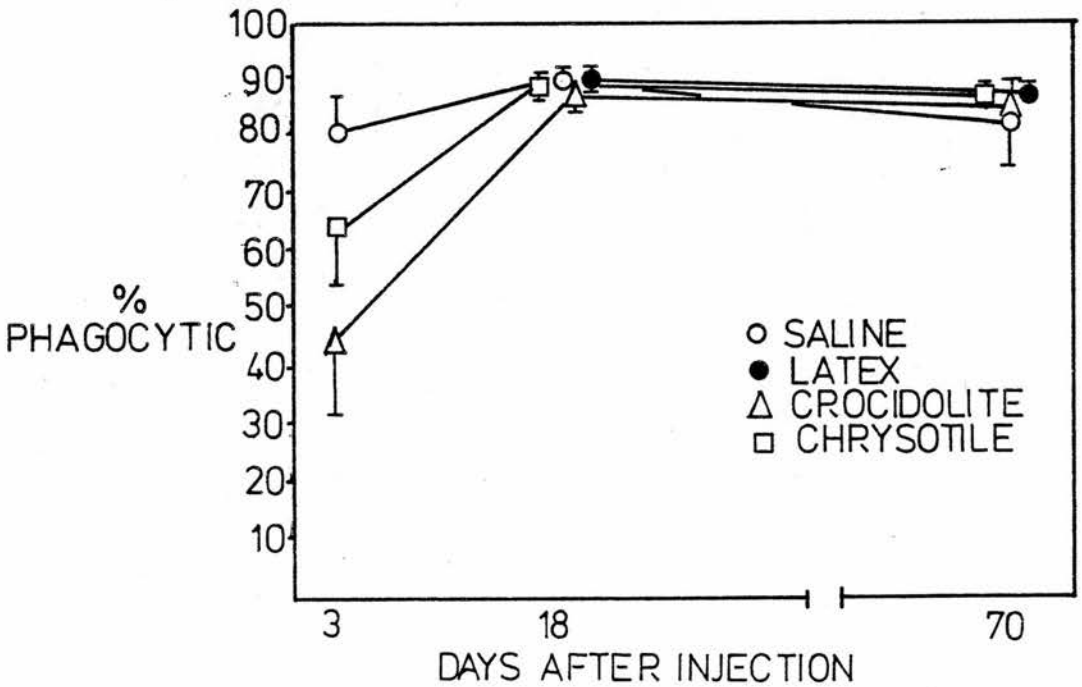


FIGURE 3.4 Mean percentage of variously induced macrophages which could phagocytose latex particles.
Significant difference between saline and crocidolite at day 3:- $P<0.05$.
Bars denote $\pm$ one standard deviation
*

* pooled data from 2 separate experiments

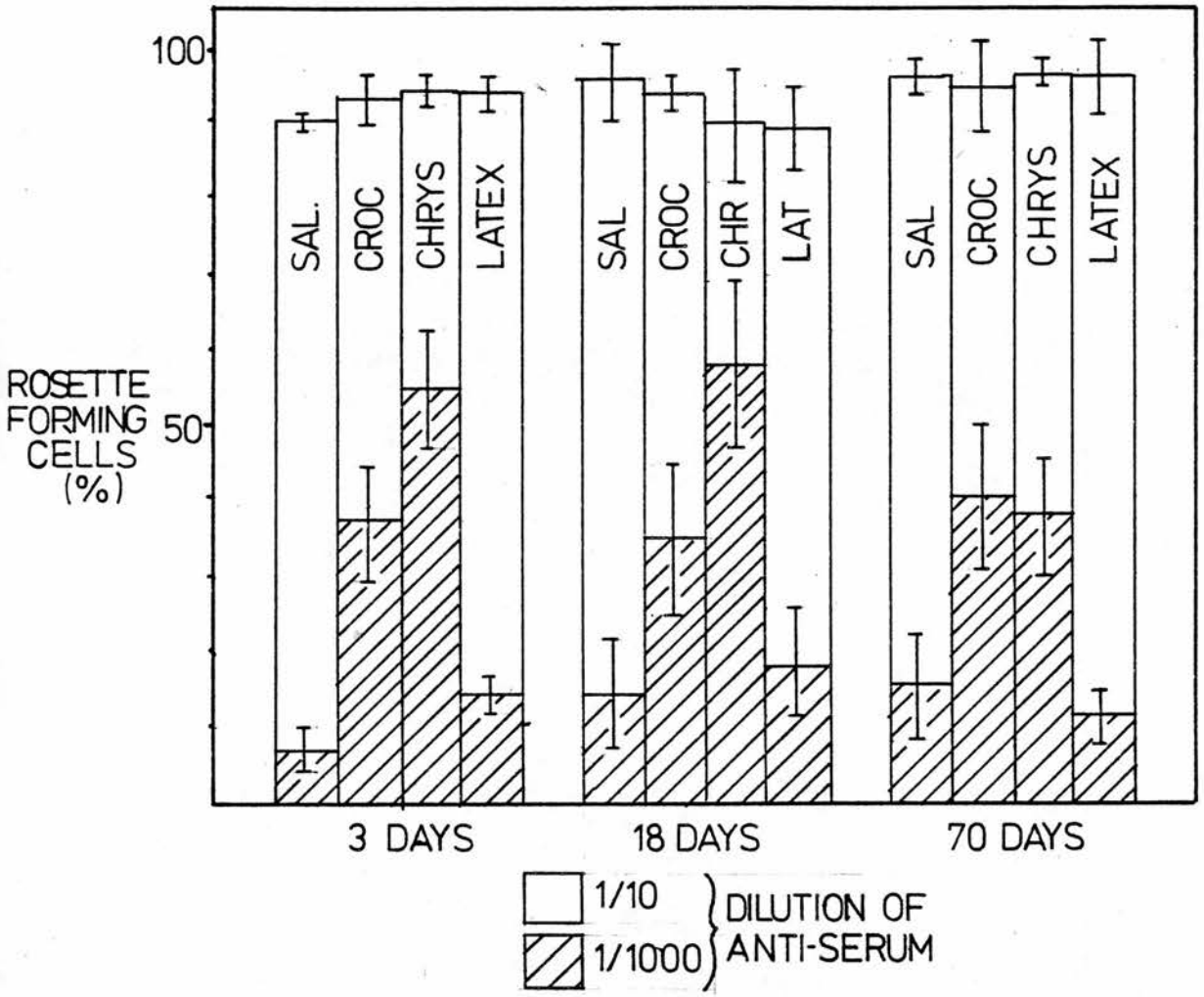


FIGURE 3.5 Mean percentage of macrophages forming Fc rosettes with SRBC's coated with 1:10 and 1:1,000 dilutions of anti-SRBC serum. Macrophages induced with various stimuli as shown. Bars denote $\pm$ one standard deviation. At all 3 time points crocidolite and chrysotile induced macrophages had a statistically significant increase in the percentage able to form rosettes with lightly sensitised SRBC's compared to saline induced macrophages. Levels of significance:- 3 day crocidolite and chrysotile $P<0.01$; 18 day crocidolite $P<0.05$, 18 day chrysotile $P<0.01$; 70 day crocidolite and chrysotile $P<0.02$. At 3 days latex also produced a significant increase ($P<0.05$) in this parameter over saline induced macrophages.

pooled data from 2 separate exp^{s}.

conditions at all time points. High affinity receptor bearing cells were detected in low proportions in saline and latex induced macrophages (on average between 5 and 20% of macrophages). The asbestos induced macrophages always had a significantly greater ($P < 0.01$ to $P < 0.05$ see Fig. 3.5 for details) percentage of cells able to form rosettes with lightly sensitized SRBC's compared to saline induced macrophages. The 3 day latex induced macrophages had a small significant ($P < 0.05$) increase in this parameter over saline induced macrophages.

In addition to forming more rosettes with lightly sensitized SRBC's the asbestos elicited macrophages also formed rosettes which had a significantly ($P < 0.001$) greater number of SRBC's/rosette than saline induced macrophages (Table 3.1).

3.2.4 Spreading

The percentage of macrophages able to spread on glass within 1 hour is shown in Fig. 3.6. At all 3 time points asbestos induced macrophages had a significantly ($P < 0.002$ to $P < 0.01$; see Fig. 3.6 for details) greater proportion of cells which spread rapidly on glass; this population was greatest at day 3 being more than 40% of all macrophages and lower at subsequent time points. Saline and latex macrophages had less than 10% of spreading macrophages at all time points, except for 3 day latex which, at 15.4% was slightly elevated.

3.2.5 Membrane 5' nucleotidase and lysosomal acid phosphatase

In addition to the three previous methods of measuring macrophage activation, a further measure was sought. The levels of the ecto-enzyme 5' nucleotidase and the lysosomal enzyme acid phosphatase are known to change with macrophage activation so these were measured in 3 day saline and chrysotile induced macrophages. Table 3.2 shows the results of the assays including appropriate controls and reveals a 7.4 fold drop in 5' nucleotidase and an 11.8 fold increase in acid phosphatase in chrysotile

TABLE 3.1 Number of SRBC's/Rosette of macrophages induced 3 days after ip injection of saline or chrysotile. Significant difference $P < 0.001$.

MACROPHAGE SOURCE	NUMBER OF SRBC's/ROSETTE
3 day saline	4.0 ± 2.3*
3 day chrysotile	8.9 ± 6.5

* $\bar{x} \pm$ sd of 50 rosette forming cells.

TABLE 3.2 Levels of 5' nucleotidase and acid phosphatase in 3 day saline and 3 day chrysotile induced macrophages.

		3 DAY INDUCED MACROPHAGES	
REACTION		SALINE	CHRYSOTILE
1	Nucleotidase	5.3 ± 0.3 *	2.4 ± 0.3
2	Acid phosphatase	1.5 ± 0.1	5.1 ± 0.9
3	Alkaline phosphatase	0.7 ± 0.2	0.7 ± 0.1
4	Spontaneous substrate hydrolysis (1)	0.2 ± 0.0	0.2 ± 0.0
5	Spontaneous substrate hydrolysis (2)	0.4 ± 0.2	0.3 ± 0.0
6	Endogenous phosphate	0.7 ± 0.1	1.0 ± 0.2
7	Nucleotidase minus controls+	5.3-1.6=3.7	2.4-1.9=0.5
8	Acid phosphatase minus controls+	1.5-1.1=0.4	5.1-0.4=4.7

* Units = μ moles phosphate/mg protein/hour.

+ for nucleotidase subtract controls 3, 4 and 6
for acid phosphatase subtract controls 5 and 6.

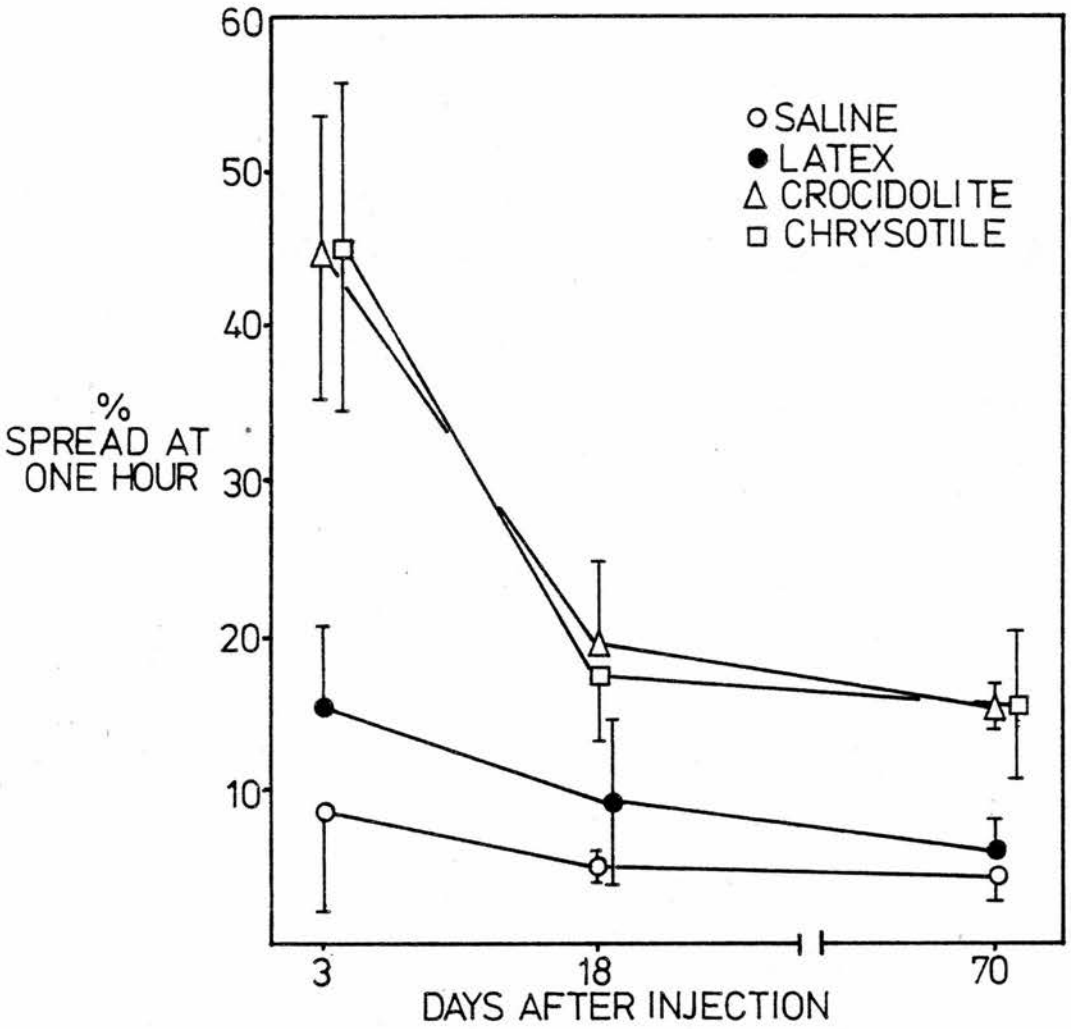


FIGURE 3.6 Mean percentage of macrophages spread on glass after 1 hour incubation at 37°C. Macrophages induced with various stimuli as shown. Bars denote ± one standard deviation. At all three time points there was a statistically significant increase in the percent spreading of asbestos induced macrophages compared to saline. Levels of significance:- 3 day crocidolite and chrysotile $P<0.01$; 18 day crocidolite and chrysotile $P<0.01$; 70 day crocidolite $P<0.002$, 70 day chrysotile $P<0.02$.

pooled data from 2 separate exp^{s}

induced macrophages compared to saline induced macrophages.

3.2.6 Morphology

The appearance of asbestos induced macrophages at various time points was compared with saline and latex induced macrophages using light and electron microscopy.

3.2.6.1 Light microscopy

One hour after being plated on to glass, stained preparations of macrophages (prepared for the spreading assay) revealed considerable differences in morphology between those induced with latex or saline and those induced with asbestos. This was true for all the time points but was particularly marked at 3 days. May-Grunwald Giemsa staining revealed saline and latex induced macrophages to be mainly small darkly staining cells (Fig. 3.7); a frill of cytoplasm indicative of spreading was, in general, absent although occasional cells were well spread and vacuolated. Asbestos induced macrophages however were typified by a sub-population, varying in proportion but always more than in the controls, of large vacuolated granular cells which had spread extensively after 1 hour on glass (Fig. 3.7). Many of these cells contained visible asbestos fibres particlularly in the case of crocidolite. The remainder of cells in the asbestos induced population were small unspread cells.

After 24 hours in culture in cRPMI there was an increase in the percentage of spread cells in the saline and latex induced populations (Fig. 3.8) but even the spread macrophages were strikingly different from asbestos induced macrophages cultured for a similar time. After 24 hours in culture the majority of asbestos induced macrophages had spread and were granular and had multiple vacuoles (Fig. 3.8).

After 3 days in culture on plastic, as revealed by low power phase contrast microscopy in Fig. 3.9, the majority of saline induced macrophages remained unspread; the majority of chrysotile induced macrophages however,

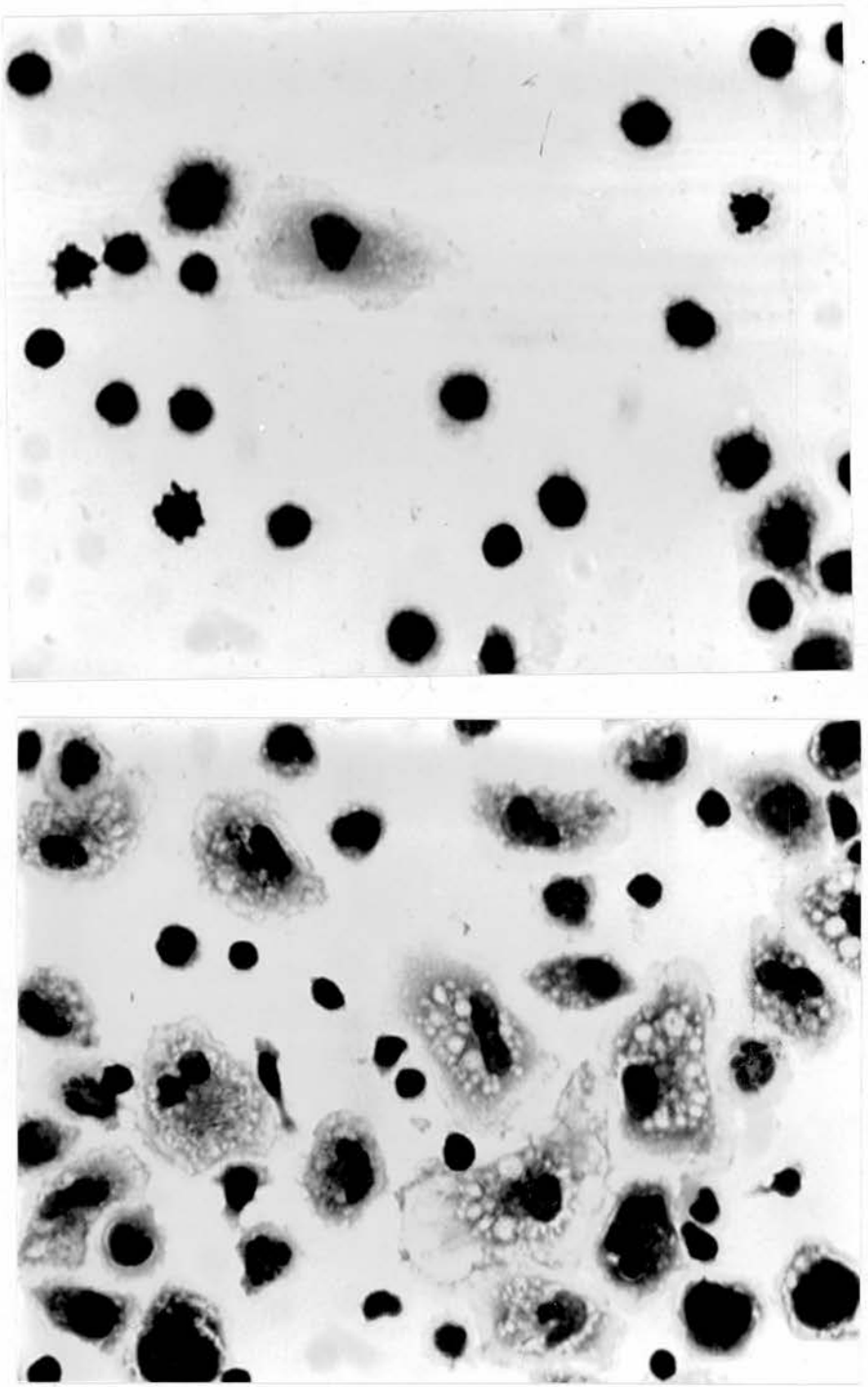


FIGURE 3.7 Light microscope view of preparations made for the macrophage spreading assay and therefore representing macrophages incubated for 1 hour at 37°C on glass.
Upper:- 3 day latex induced macrophages.
Lower 3 day crocidolite induced macrophages.
May-Grunwald Giemsa stain.

Mag. x 450

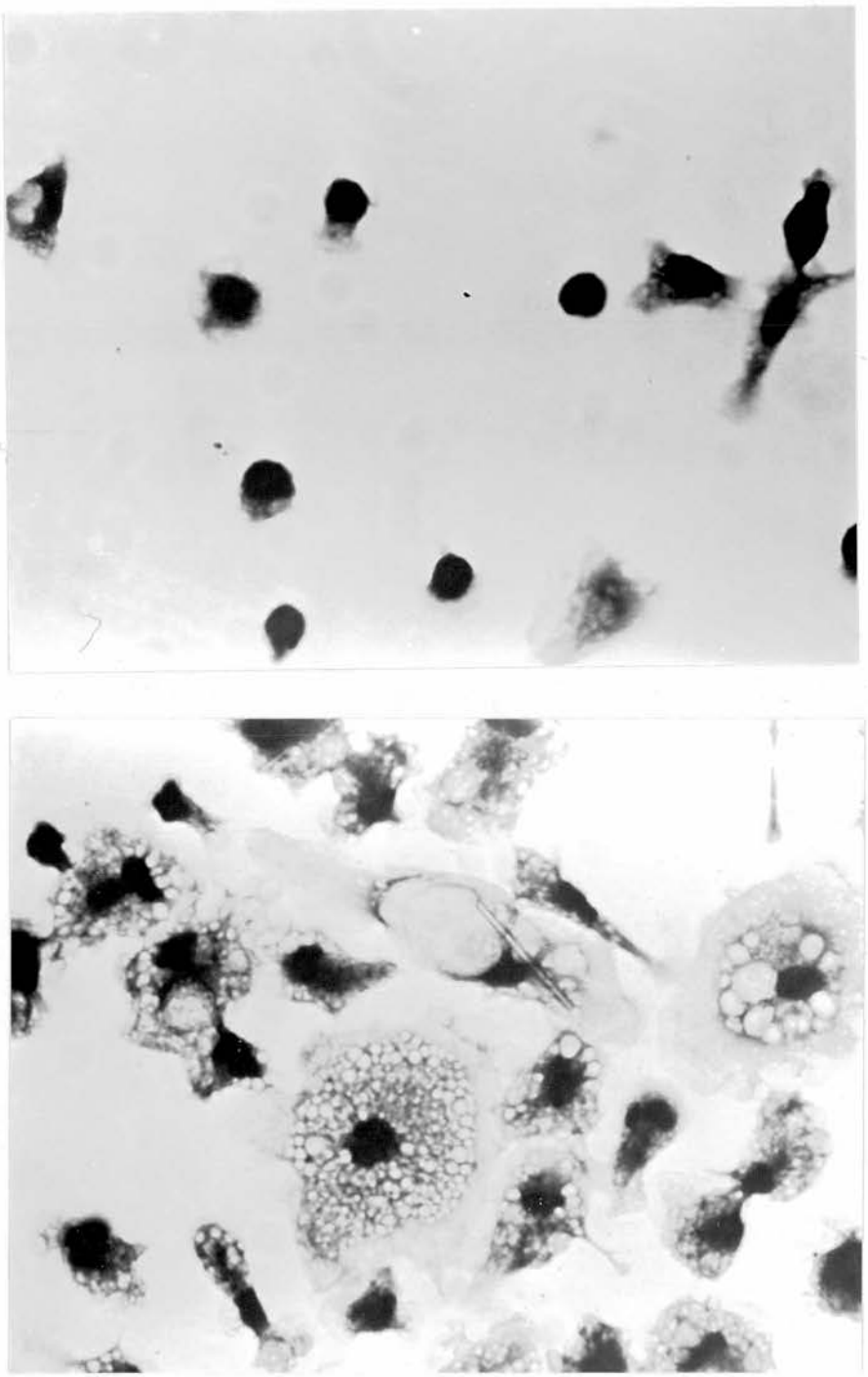


FIGURE 3.8 Macrophages incubated for 24 hours.
Upper:- 3 day latex induced macrophages;
Lower:- crocidolite induced macrophages.
May-Grunwald Giemsa stain.

Mag. x 450

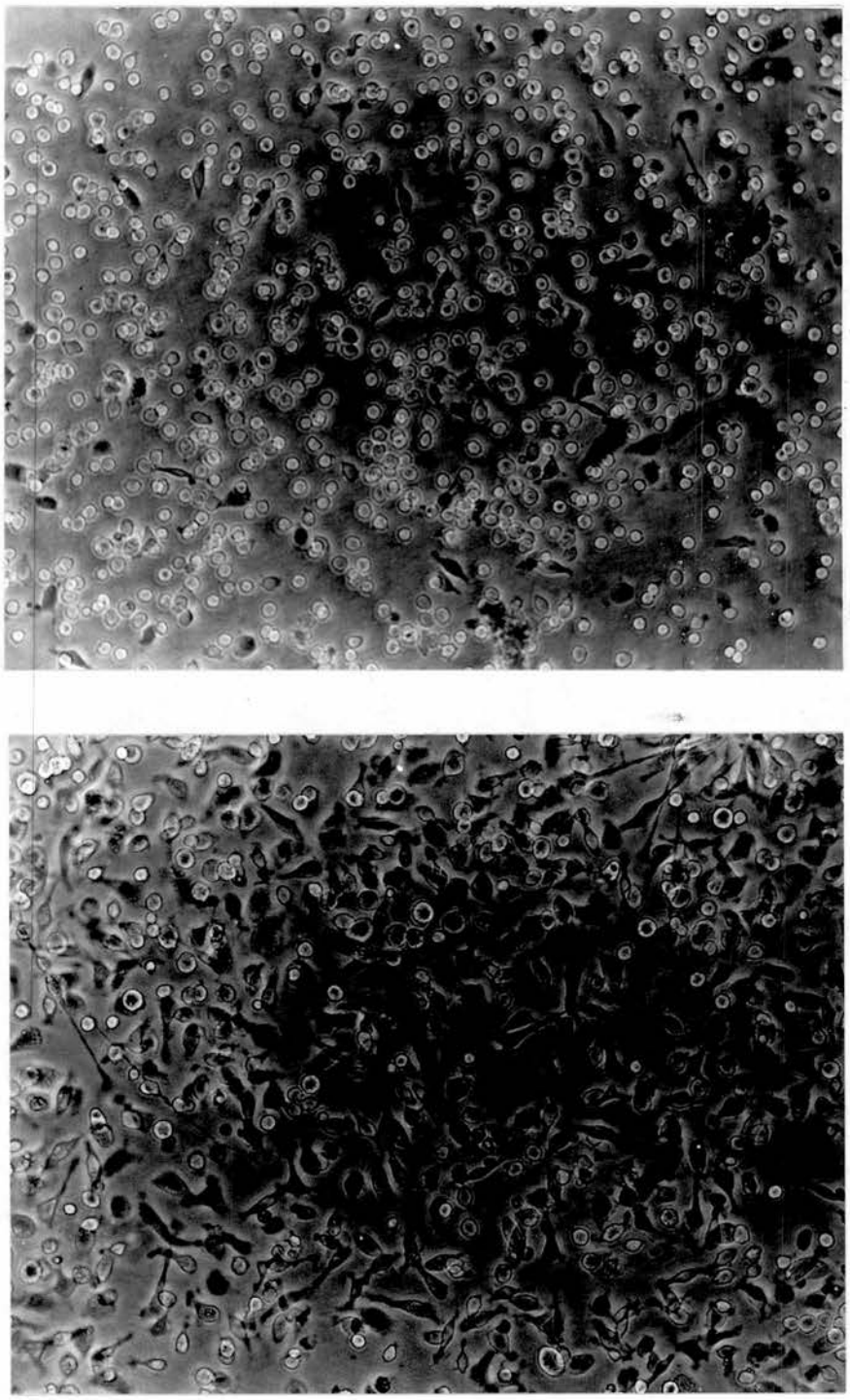


FIGURE 3.9 Low power phase contrast view of macrophages after 3 days in culture on plastic.
Upper:- saline induced;
Lower:- crocidolite induced.

Mag. x 150

were spread out on the plastic although a small sub-population remained unspread.

3.2.6.2 Transmission Electron Microscopy (TEM)

The differences between asbestos induced and control macrophages evident at light microscopy were confirmed by TEM. Fig. 3.10 shows 18 day latex induced PEC containing predominantly macrophages compared to 18 day chrysotile PEC. In the case of the chrysotile PEC the induced macrophages are increased in size and have much increased membrane activity evident as complex infoldings of the surface.

Sections of 3 day chrysotile induced PEC were not, in general of good quality due to large amounts of chrysotile which interfered with good sectioning; some sections of acceptable quality were, however, obtained. Fig. 3.11 shows a low power view of a 3 day chrysotile induced macrophage illustrating the convoluted nature of the plasma membrane. Fig. 3.11 also shows a grazing section of such a cell to emphasise the convoluted nature of the surface.

Fig. 3.12 shows a 70 day saline induced macrophage which is normal in appearance compared with a 70 day chrysotile induced macrophage; the latter has a large number of mitochondria and granules and possesses a highly developed Golgi/vesicle system. The overall impression of the chrysotile induced macrophages was, therefore, consistent with the increased secretory status and metabolism of the activated macrophage.

Fig. 3.13 is a high power view of a 70 day chrysotile induced macrophage with chrysotile fibrils present in a phagolysosome confirming that the resident macrophages, $2\frac{1}{2}$ months after injection of asbestos, are still handling asbestos through their vacuolar system.

Fig. 3.14 is a low power view of a multi-nucleate giant cell from 18 day chrysotile PEC and chrysotile is visible lying free in the cytoplasm and also membrane bound. Multinucleate giant cells were present in low

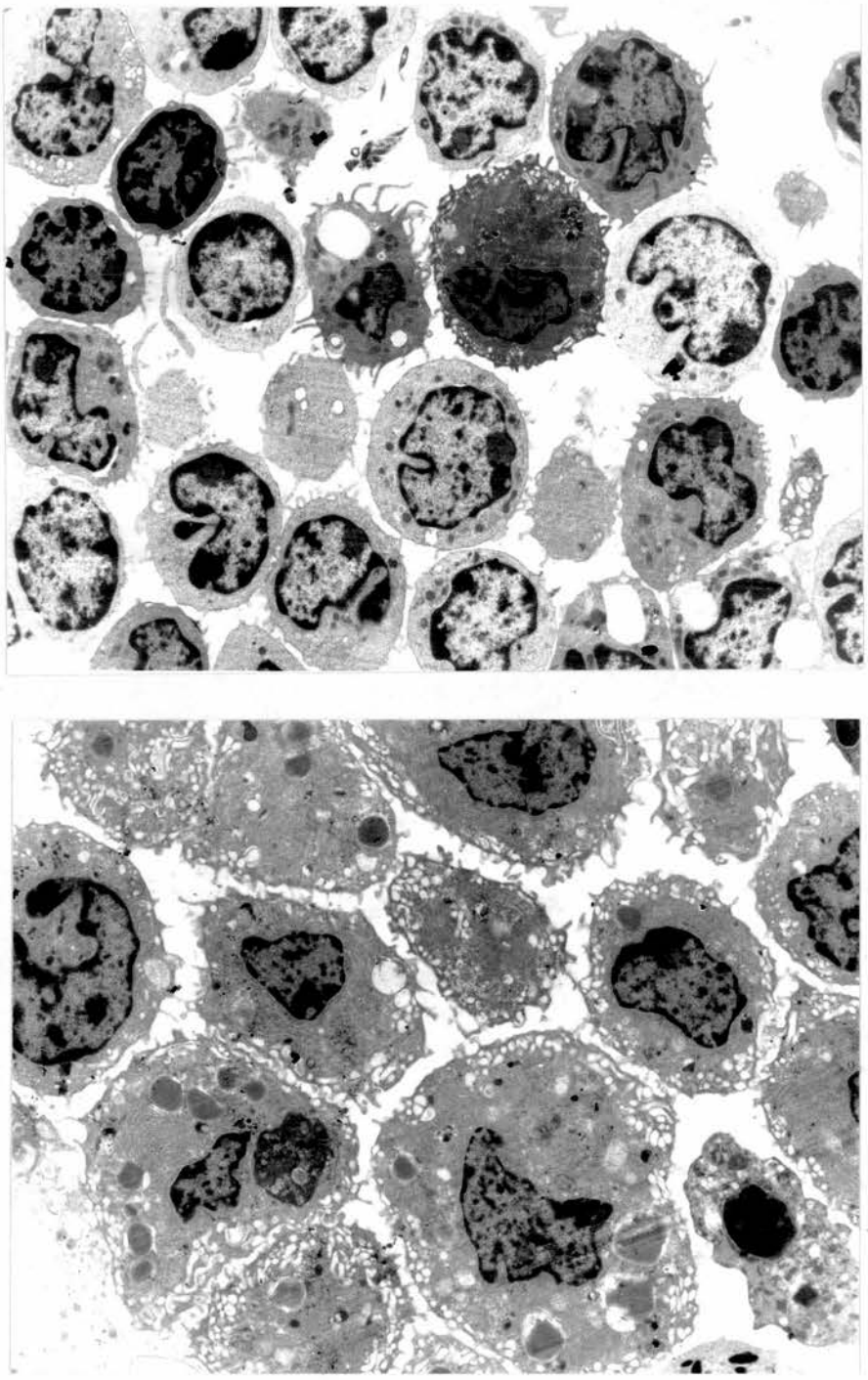


FIGURE 3.10 Transmission electron micrographs of 18 day induced macrophages.
Upper:- latex induced;
Lower:- chrysotile induced.

Mag. x 3,000

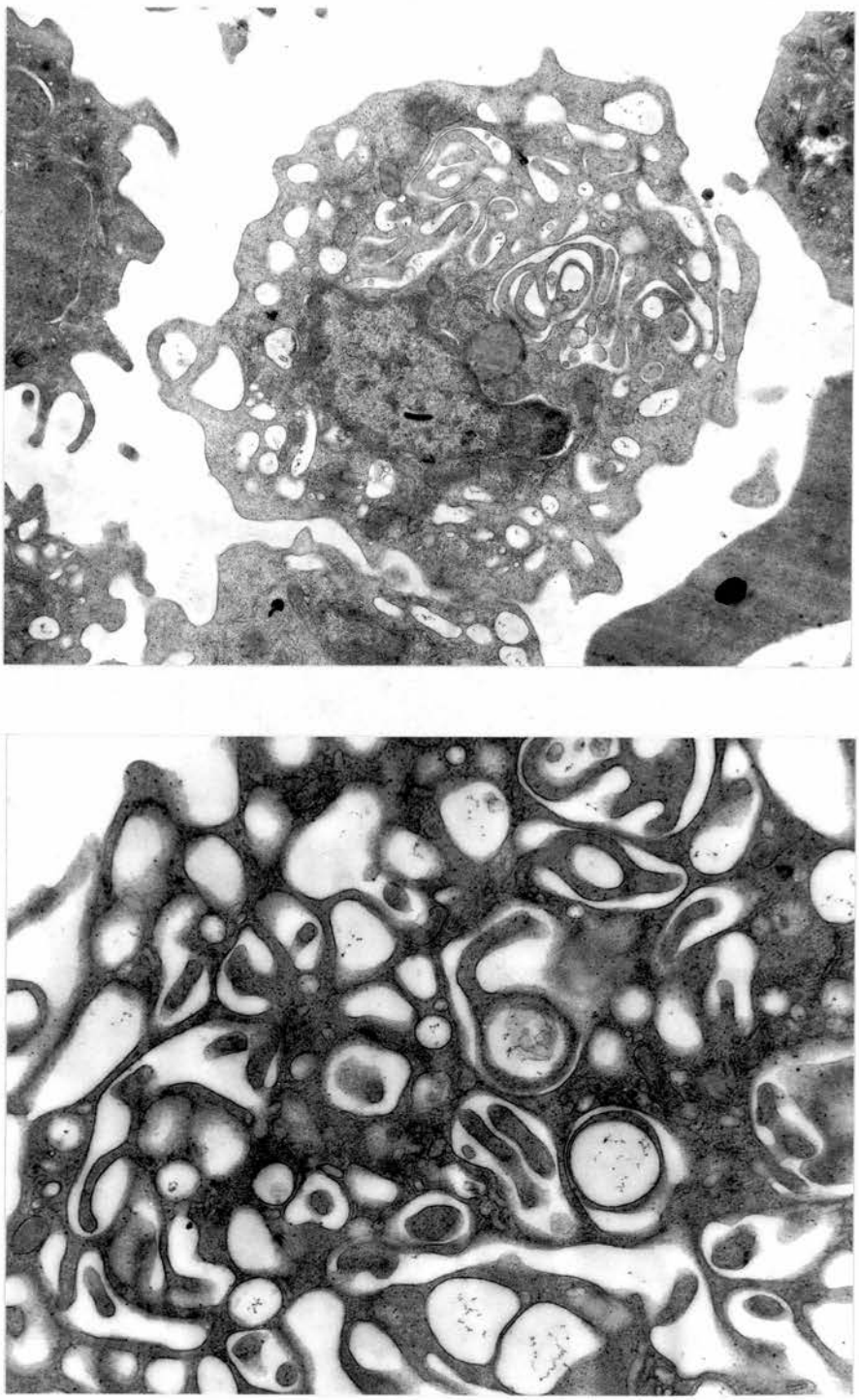


FIGURE 3.11 3 day chrysotile induced macrophages to show convolutions of cell membrane.
Upper:- low power view Mag. x 5,000
Lower:- high power grazing section of a macrophage to show complex nature of surface infoldings Mag. x 12,000

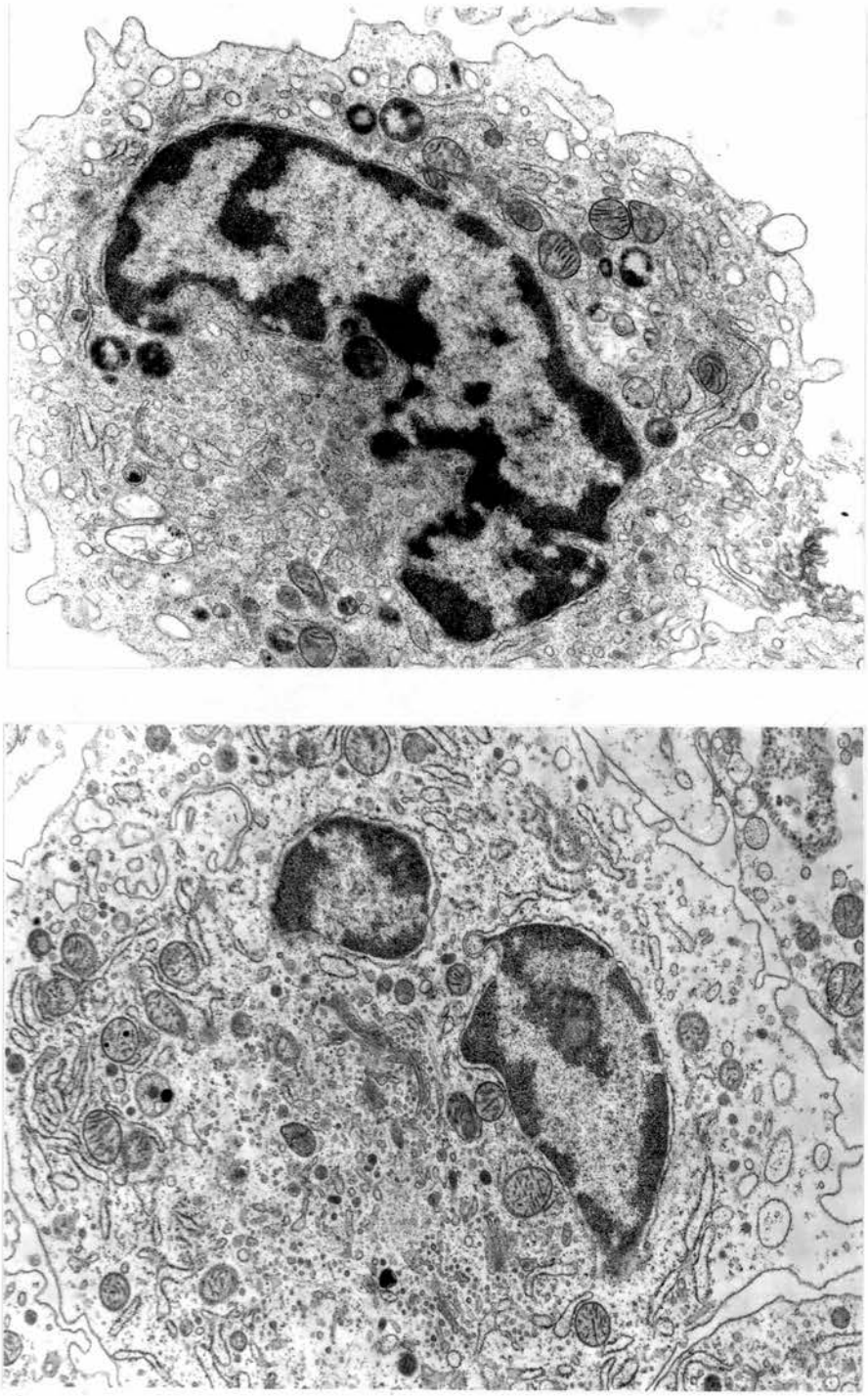


FIGURE 3.12 70 day induced macrophages.
Upper - saline induced;
Lower - chrysotile induced.

Mag. x 7,500

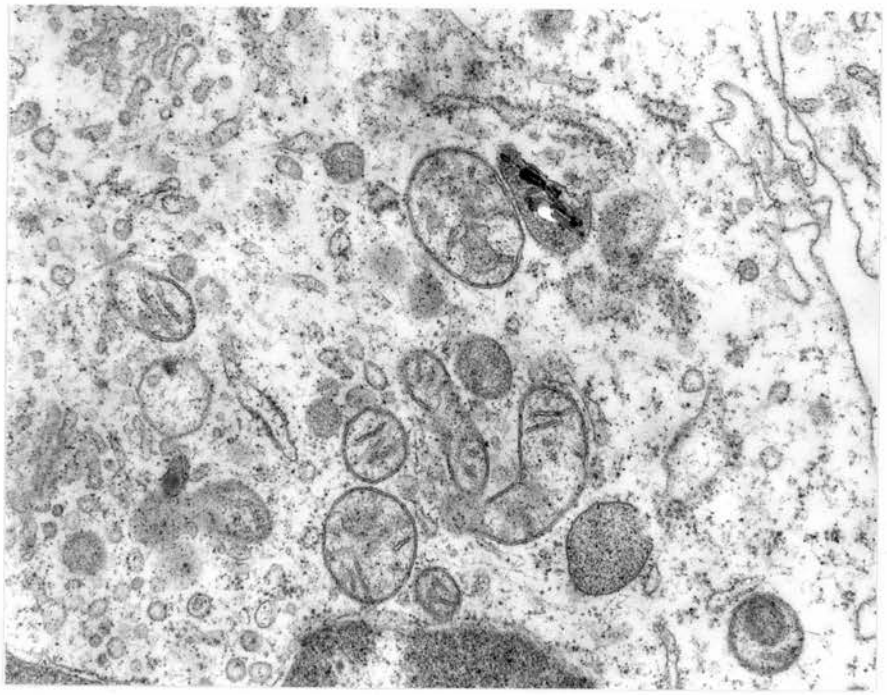


FIGURE 3.13 High power view of a 70 day chrysotile induced macrophage showing chrysotile fibrils in a lysosome.

Mag. x 12,500

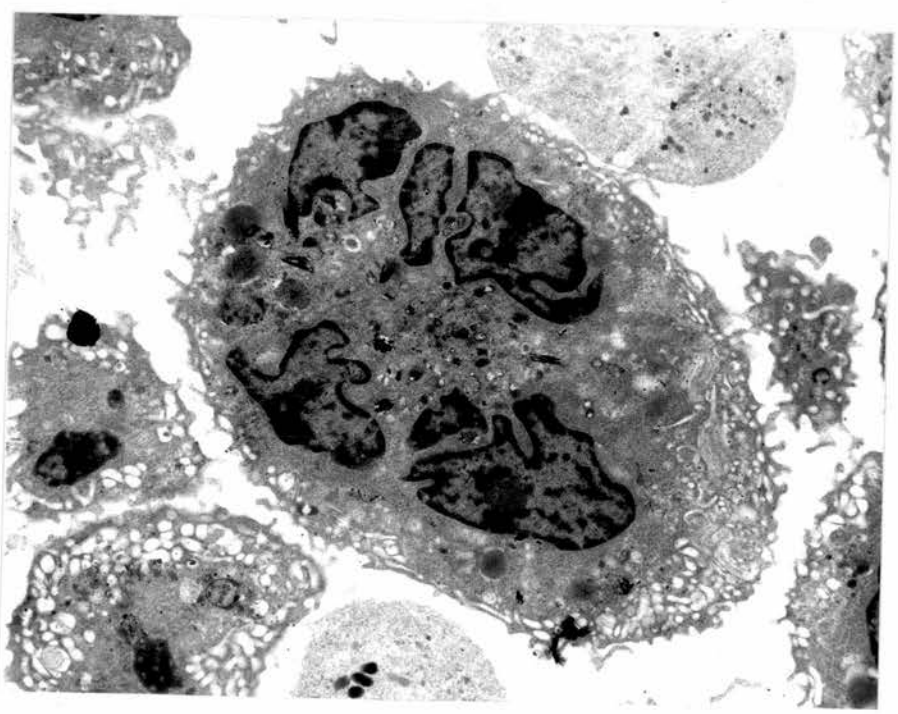


FIGURE 3.14 Multinucleate giant cell in peritoneal exudate cells obtained 18 days after chrysotile injection.

Mag. x 4,500

amounts (< 5%) in all asbestos induced PEC.

3.2.6.3 Scanning Electron Microscopy (SEM)

All SEM preparations of macrophages were prepared by incubating PEC on glass coverslips for 1 hour in cRPMI then fixing so they are directly comparable with spreading assays.

Fig. 3.15 (upper) shows two typical 3 day saline induced macrophages with shallow surface ruffles; although attached to the coverslip the cells have not spread. As shown in Fig. 3.15 (lower) a sub-population of saline induced macrophages did spread but they were very much in the minority as would be expected from the spreading assay results. Fig. 3.16 (upper) is a low power view of 3 day chrysotile induced macrophages and reveals heterogeneity in the population with regard to spreading activity with unspread, partially spread and spread cells being represented. As shown in Fig. 3.16 (lower) a representative 3 day chrysotile induced macrophage is larger than a saline induced macrophage, has more surface ruffles and is more spread. Fig. 3.17 shows a high power view of a typical 18 day chrysotile and saline induced macrophage and reveals the more numerous and complex membrane ruffling of the chrysotile induced macrophages.

3.3 Interaction of asbestos and asbestos activated macrophages with an experimental tumour in vivo and in vitro

3.3.1 Tumour cell cytotoxic activity of asbestos induced macrophages

3.3.1.1 Thymidine release assay

Since asbestos induced peritoneal macrophages were found to be activated by several criteria (3.2) experiments were carried out to determine whether these macrophages were tumouricidally activated.

Fig. 3.18 shows the percent specific tumour cell cytotoxicity due to crocidolite and chrysotile induced macrophages for 3 different

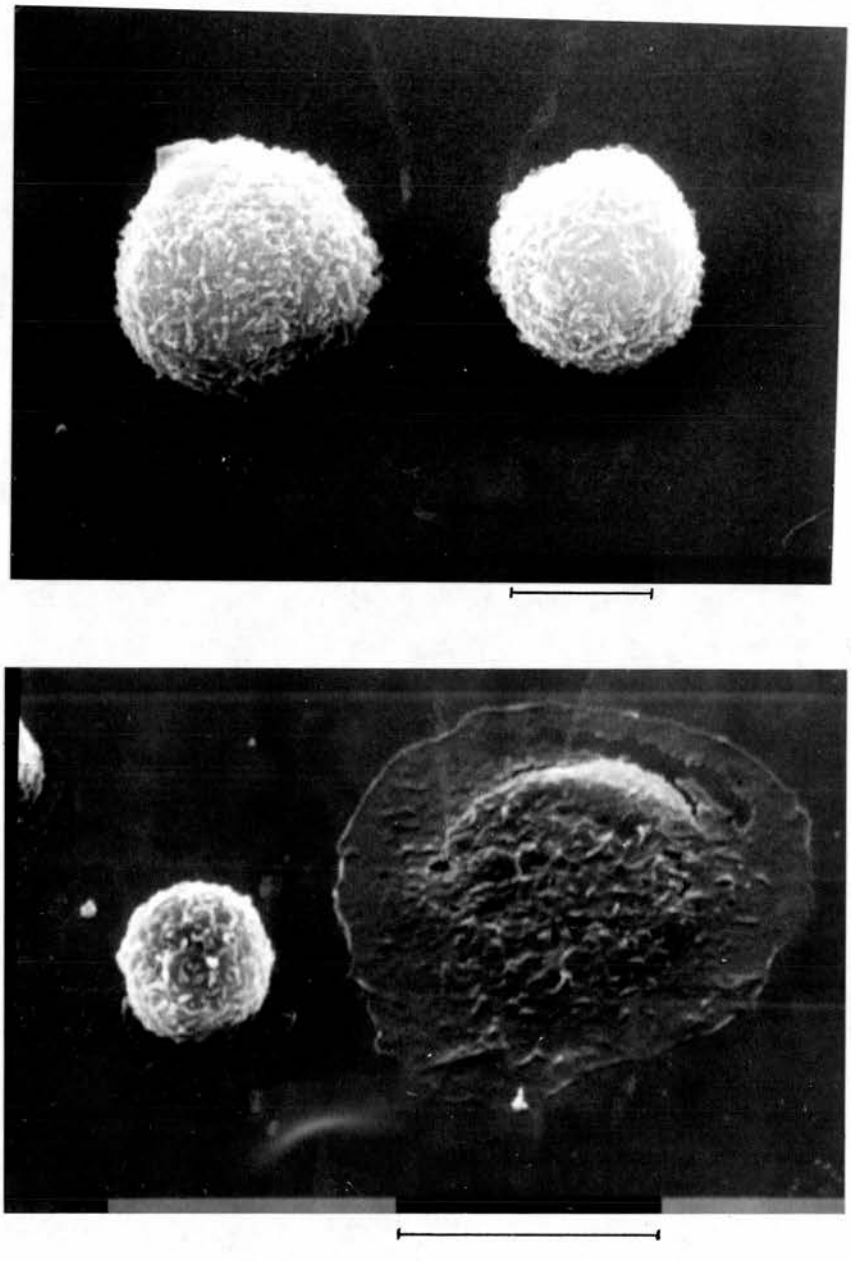
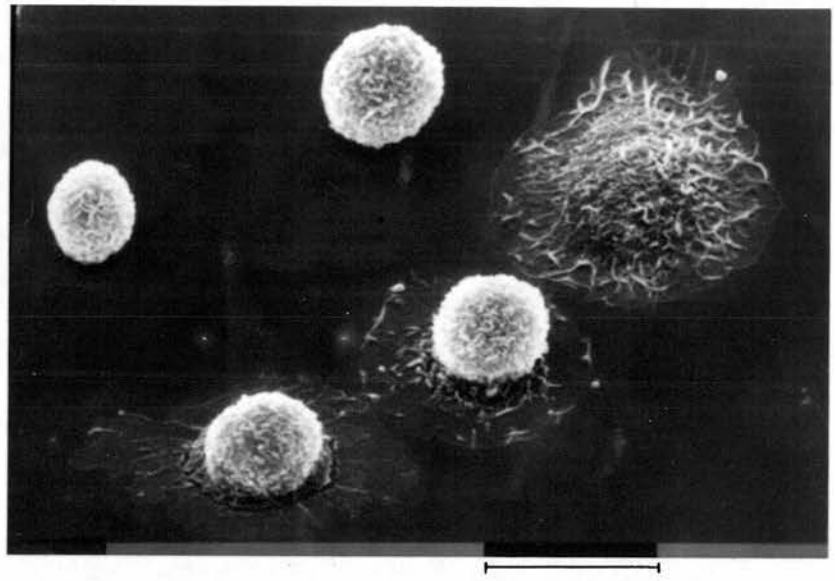


FIGURE 3.15 Scanning electron micrographs of 3 day saline induced macrophages.
Upper - 2 typical unspread macrophages. Marker line = 4 μm.
Lower - 1 typical unspread macrophage and a well spread macrophage. Marker line = 10 μm.



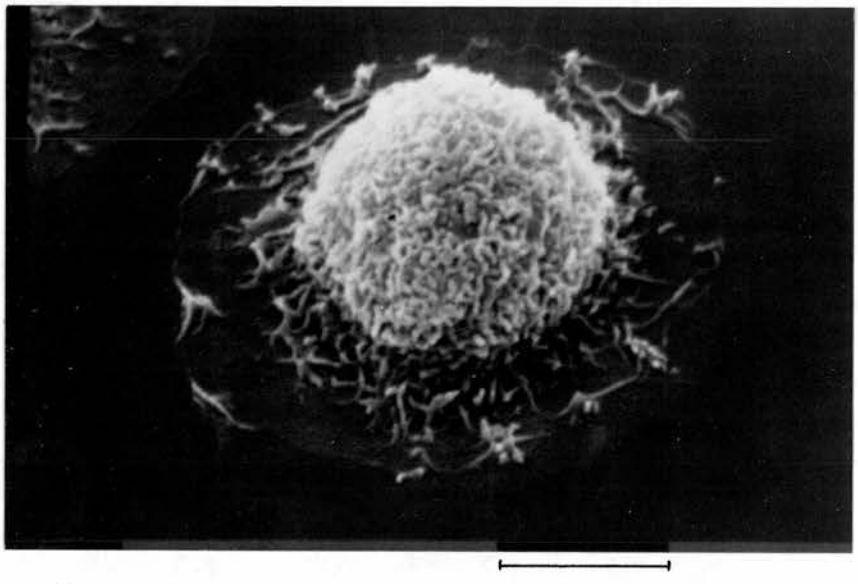


FIGURE 3.16 3 day chrysotile induced macrophages.
Upper - typical heterogeneity of macrophage spreading. Marker line = 10 μm.
Lower - higher power view of a typical spread macrophage. Line marker = 4 μm.

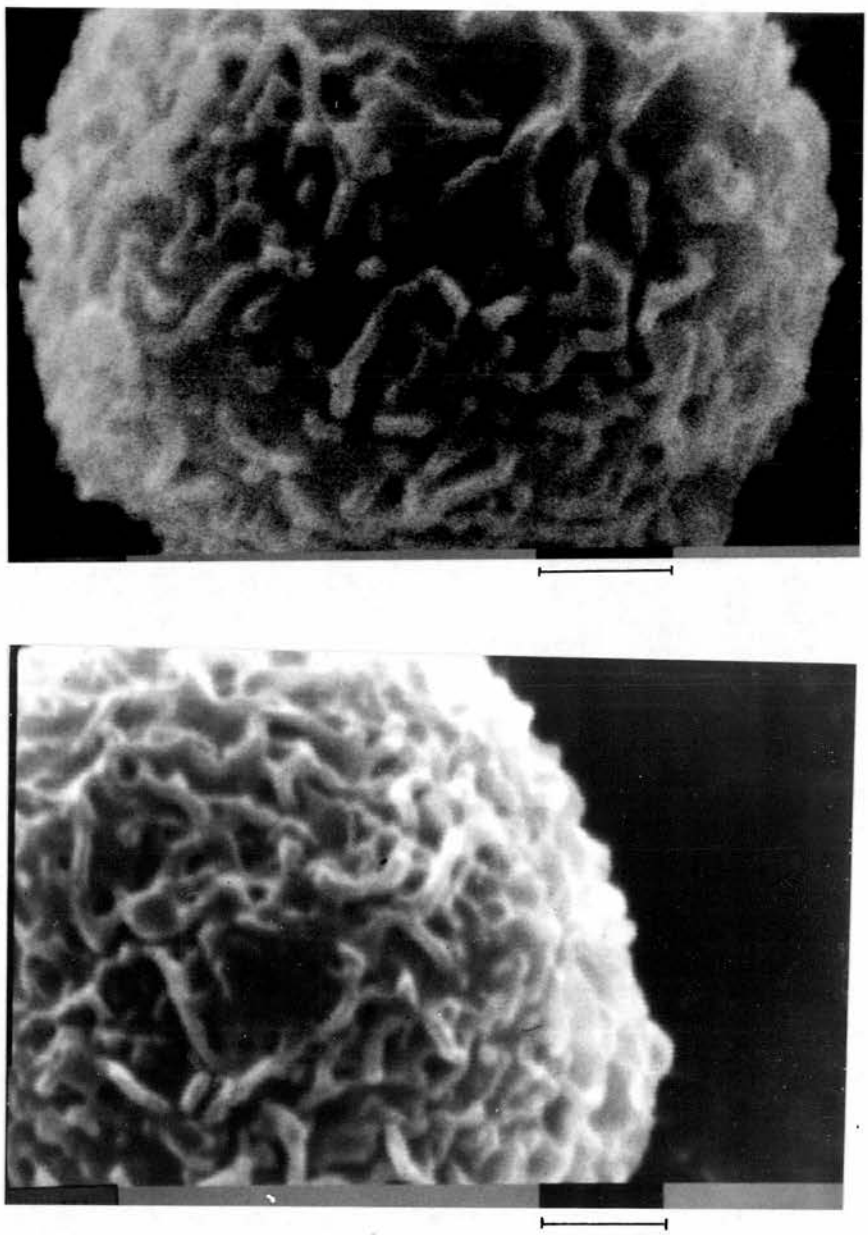


FIGURE 3.17 High power views of 18 day induced macrophages.
Upper - saline induced. Lower - chrysotile induced. Line marker = 1 μm.

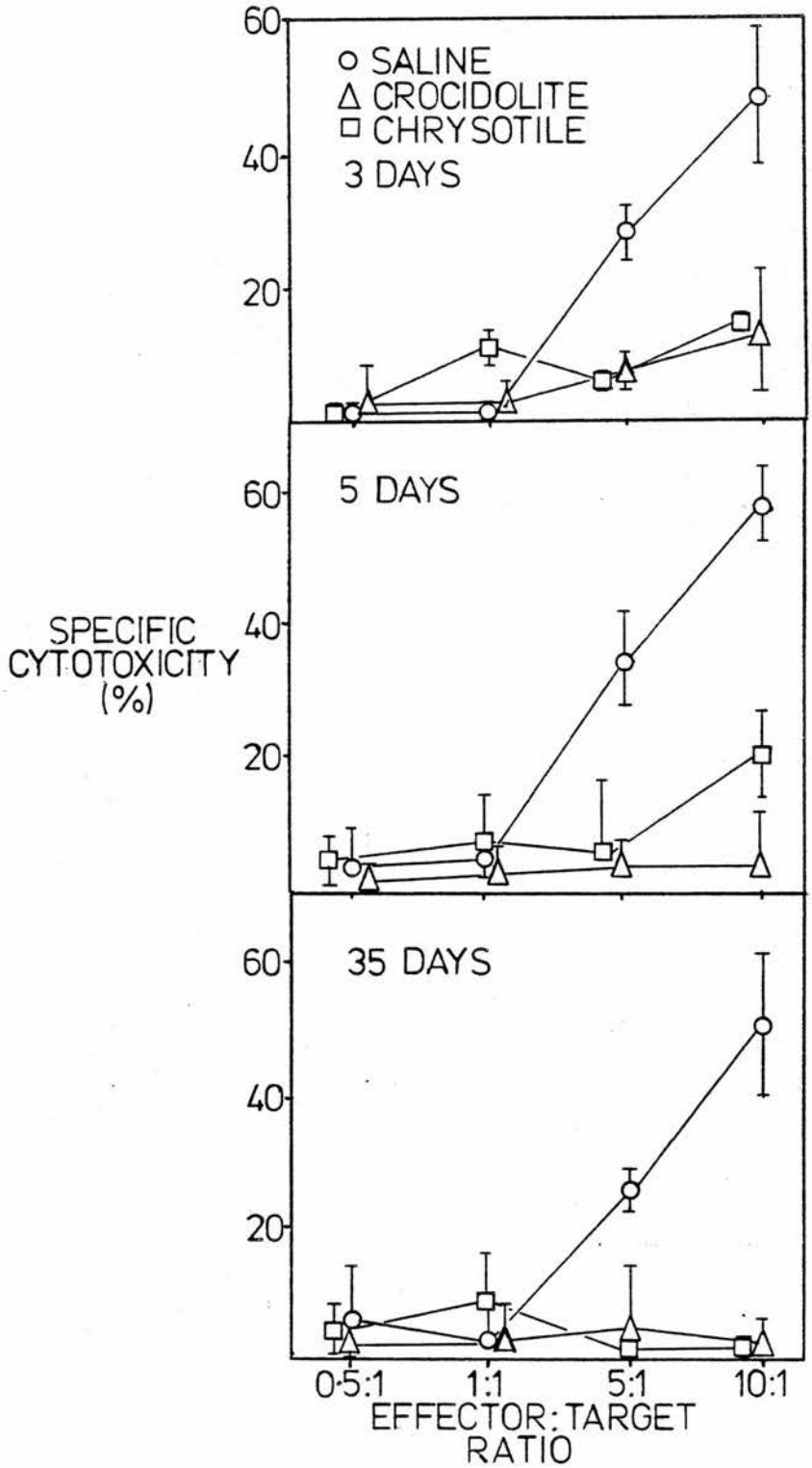


FIGURE 3.18 Mean percent specific cytotoxicity to tumour cells of macrophages induced by intraperitoneal injection of crocidolite (△), chrysotile (□) at 3, 5 and 35 days, 5 day *C. parvum* (○) induced macrophages present as positive controls. Bars denote ± one standard deviation.
3 separate exp^ts for each time point

experiments carried out at the 3 different time points. In this case 35 days was used instead of 70 days since the absence of tumouricidal activity at 5 days made it unlikely that this property would develop at all. A positive macrophage tumour cell cytotoxic control of C. parvum activated peritoneal macrophages showed a mean toxicity, towards the tumour cell targets for all experiments, of 24.7% at 5:1 and 54.0% at 10:1 effector:target ratios. Specific cytotoxicity due to asbestos was low at all times mostly remaining below 10% and only once exceeding 20% (with 5 day chrysotile at 10:1 ratio) and was considered negligible.

3.3.1.2 Visual evidence

In order to visually demonstrate the outcome of a tumour cell killing assay, in the manner of Hibbs et al (1980), an assay was scaled up into 8 well plates using 3 day saline, crocidolite, chrysotile and C. parvum macrophages at 10:1 with tumour cells. After 2 days the plates were stained with May-Grunwald Giemsa and photographed. Fig. 3.19 shows a stained plate where the density of staining of tumour cells is directly proportional to their number. The order of density of staining is control > saline > crocidolite > chrysotile > C. parvum. Although not quantitative there is a suggestion that chrysotile induced macrophages have slightly more cytotoxic potential than crocidolite induced macrophages.

3.3.1.3 Electron microscopy of interactions between asbestos activated macrophages and tumour cells

SEM When asbestos activated macrophages were found not to be appreciably tumouricidal compared to C. parvum activated macrophages in the thymidine release assay, electron microscopy was carried out to determine whether the ability of asbestos activated macrophages to physically interact with the tumour cells was impaired. Macrophages were incubated with tumour cells at 10:1 ratio for 2 days before

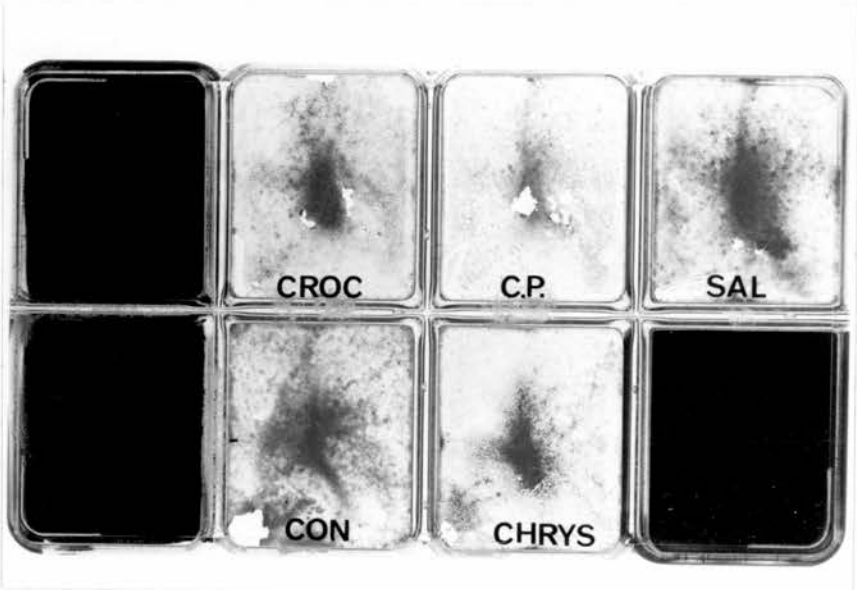


FIGURE 3.19 Scaled up macrophage/tumour cell killing assay using saline, crocidolite, chrysotile and C. parvum induced macrophages stained to show the density of tumour cells at the end of the assay period.

Mag. x $\frac{3}{4}$

processing for SEM.

Fig. 3.20 shows non-tumouricidal 3 day saline induced macrophages which have been incubated with CCH_1 tumour cells; they have not spread on the surface of the tumour cells as shown also by the higher power view in Fig. 3.20. Fig. 3.21 shows tumouricidal C. parvum activated macrophages in contact with CCH_1 tumour cells which are undergoing bleb formation and lysis. It is particularly notable that the macrophages are spread out on the tumour cell and there is therefore a large area of membrane contact.

Fig. 3.22 shows chrysotile induced macrophages in contact with a tumour cell; it is evident from photographs taken at low and high power that these macrophages are not spread but have remained with a small area of membrane contact.

Thus in the case of saline and chrysotile induced macrophages, which were not tumouricidal, there was minimal contact between the macrophage and the tumour cells while in the case of the tumouricidal C. parvum activated macrophages there was spreading on to the tumour cell surface with a resultant large area of membrane contact. It is possible that this physical difference in macrophage/tumour cell interaction could underlie the difference in tumouricidal potential of the macrophage populations.

3.3.2 In vitro tumour cell cytostatic activity of asbestos induced macrophage supernatants

The results shown in Fig. 3.23 were obtained from 5 separate experiments where 24 hour supernatants from 3 day asbestos and control macrophages were tested in an assay of tumour cell proliferation in vitro. The large standard errors reveal a considerable variation in response of the tumour cells to the supernatants. There were no significant inhibitory effects of any of the macrophage supernatants on CCH_1 tumour cell proliferation although there is a suggestion that chrysotile macro-

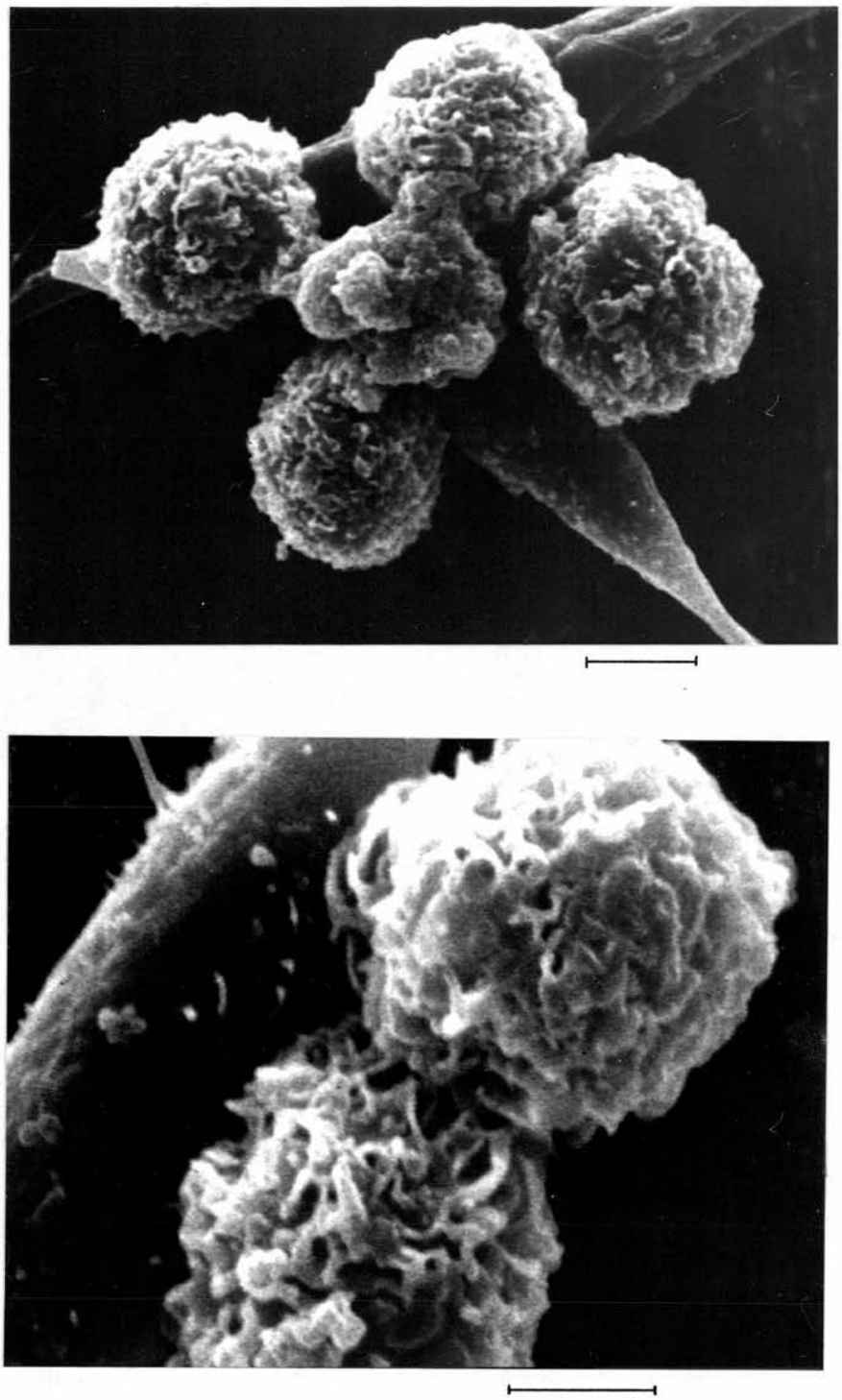


FIGURE 3.20 3 day saline induced macrophages incubated with tumour cells for 2 days.
Marker bars - upper 4 μm, lower 4 μm.

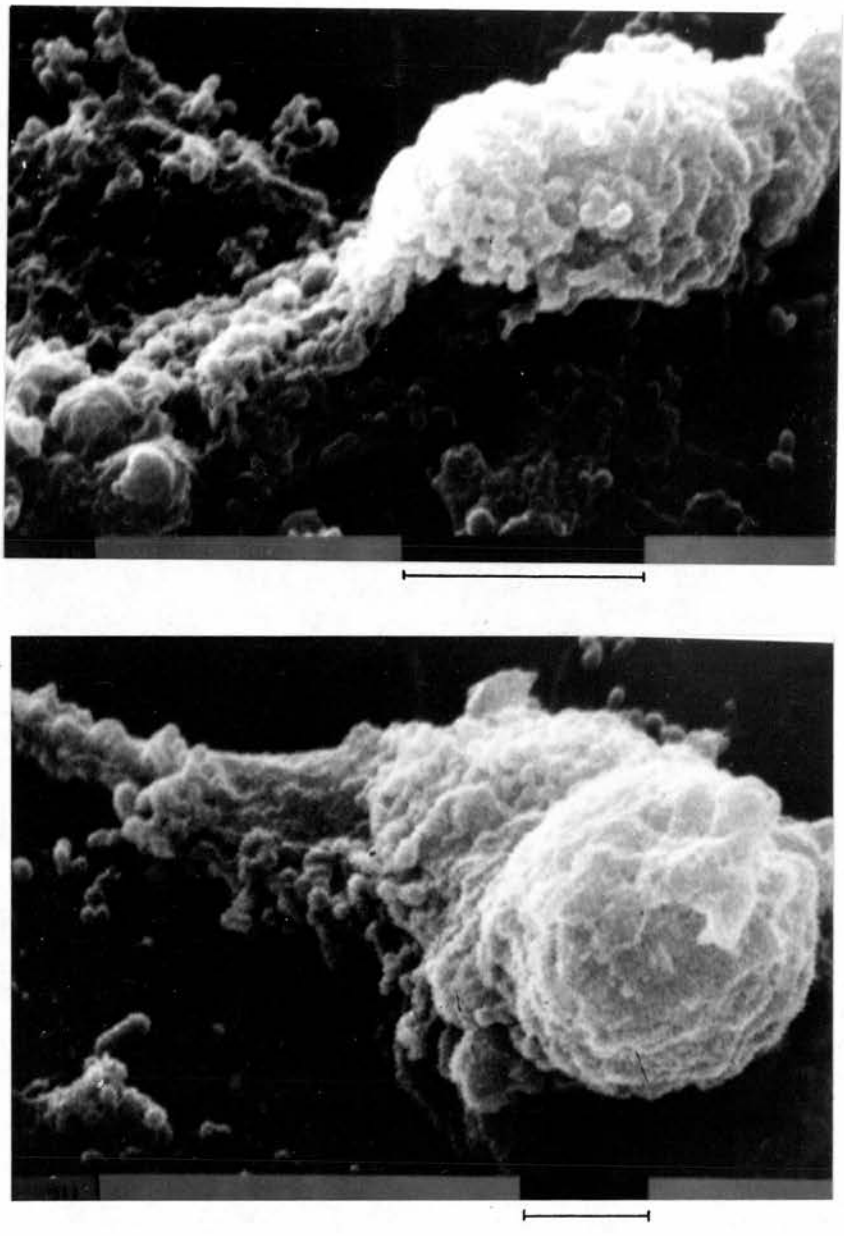
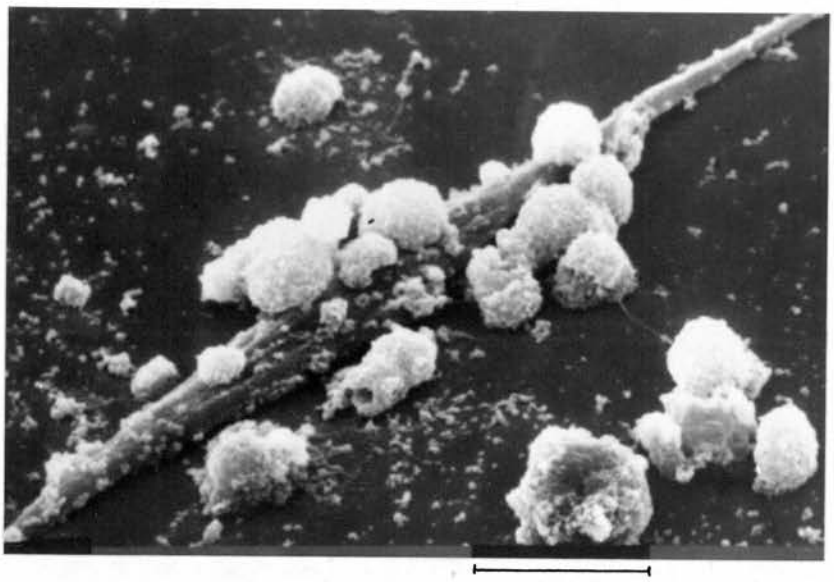


FIGURE 3.21 C. parvum induced macrophages interacting with tumour cells after 2 days co-culture. Note extensive spreading of macrophages on tumour cell surface.
Marker line - upper 4µm, lower 2 µm.



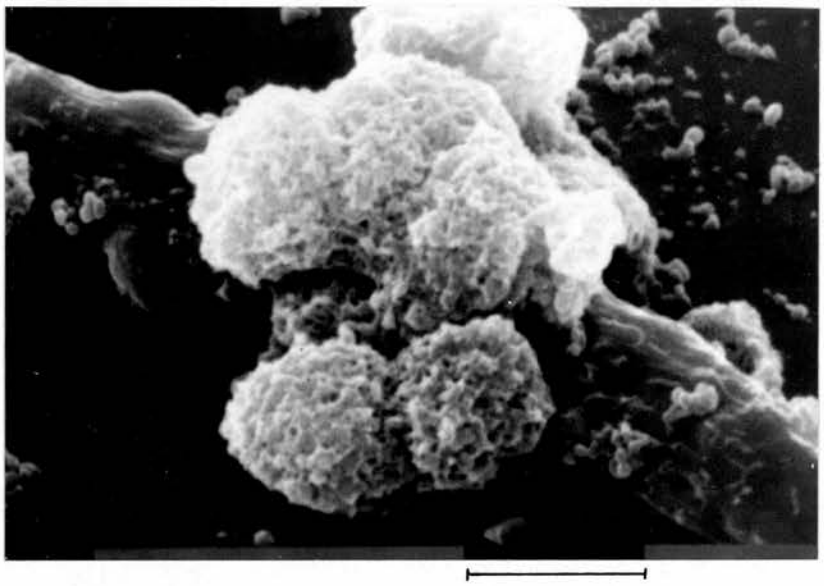


FIGURE 3.22 Chrysotile induced macrophages interacting with tumour cells after 2 days co-culture. Marker line - upper 10 μm, lower 4 μm.

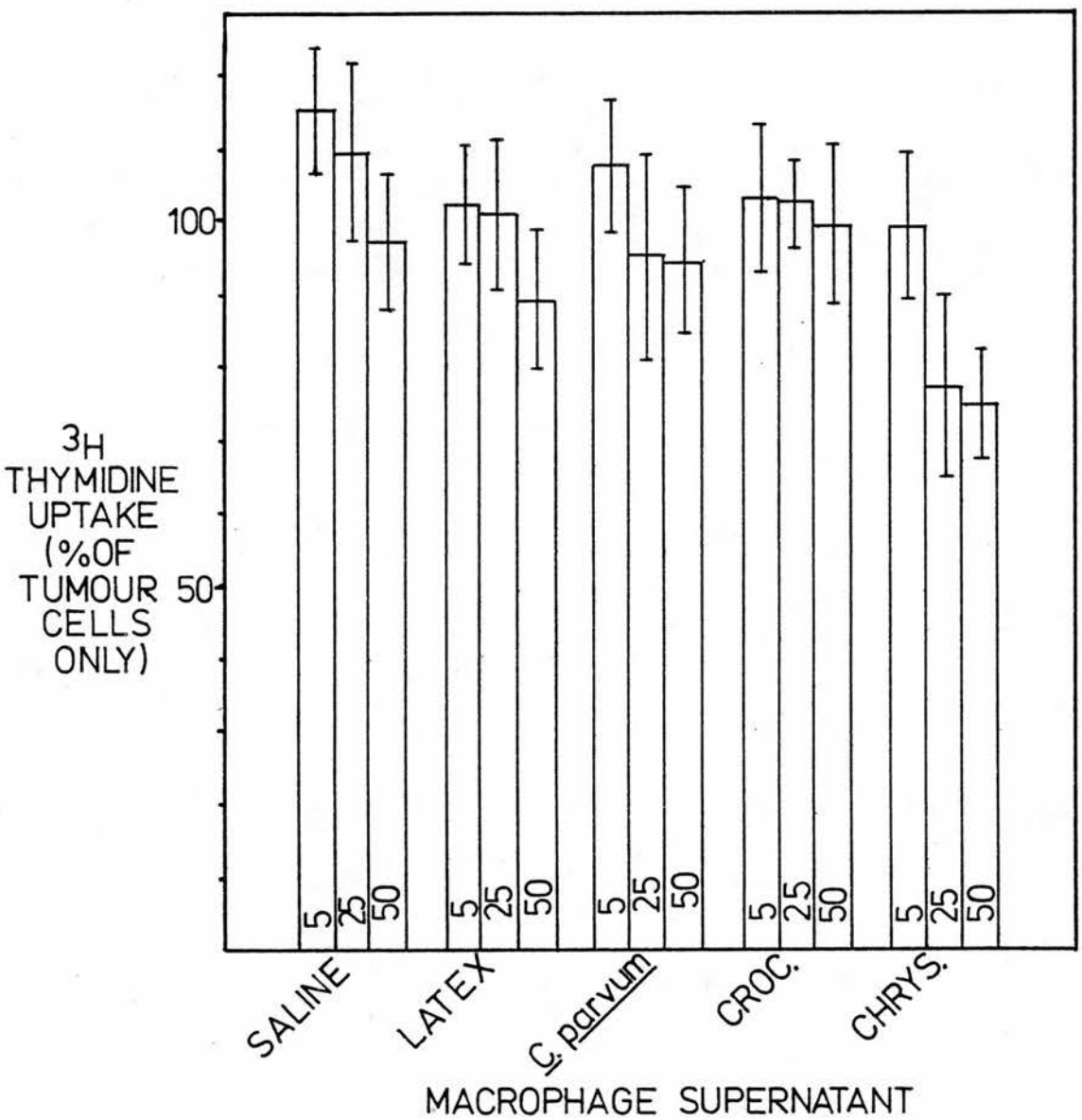


FIGURE 3.23 **Effect of various macrophage supernatants on uptake of thymidine by tumour cells. Mean percent uptake, in the presence of 5, 25 or 50% macrophage supernatant is shown, compared to uptake by tumour cells alone. Bars denote ± one standard error.**
data from 5 separate exp^{s}

phage supernatants could have some inhibitory activity.

It was notable that C. parvum activated macrophage supernatants were not cytostatic although the macrophages themselves were cytotoxic in thymidine release assays. This reveals that a diffusible cytotoxin was not responsible for the cytotoxicity of the C. parvum activated macrophages.

3.3.3 Experiments to detect the in vivo effects of asbestos and asbestos activated macrophages on tumour growth

These experiments were carried out using the CCH_1 subcutaneous tumour model in mice and attempts were made to detect tumour growth inhibition by asbestos or asbestos induced PEC.

3.3.3.1 Effects of intraperitoneal asbestos injection on subcutaneous tumour growth

(i) 5 mg chrysotile 3 days after 10^6 s/c tumour cells

Fig. 3.24 shows that ip injection of 5 mg chrysotile 3 days after tumour cell inoculation resulted in significant (Day 13 $P<0.01$; Day 17 $P<0.001$) reduction in tumour size compared to saline injected mice. Fig. 3.25 shows the same experiment repeated to include a latex injected, phagocytic stimulation, control group. Once again chrysotile caused significant (Day 10 $P<0.02$; Day 13 and 17 $P<0.002$) reductions in tumour size compared to the saline control; latex injection caused a significant ($P<0.01$) reduction in tumour size on Day 10 compared to the saline controls but by Day 13 and 17 the latex injected mice had tumour sizes not significantly different from the saline injected controls.

(ii) 5 and 20 mg chrysotile 3 days after $5x10^2$ s/c tumour cells

In an attempt to increase the effect of chrysotile in inhibiting tumour growth 5 and 20 mg of chrysotile were given intraperitoneally on day 3 following a reduced tumour cell inoculum of $5x10^2$ tumour cells (Fig. 3.26). No significant differences were found at any of the time

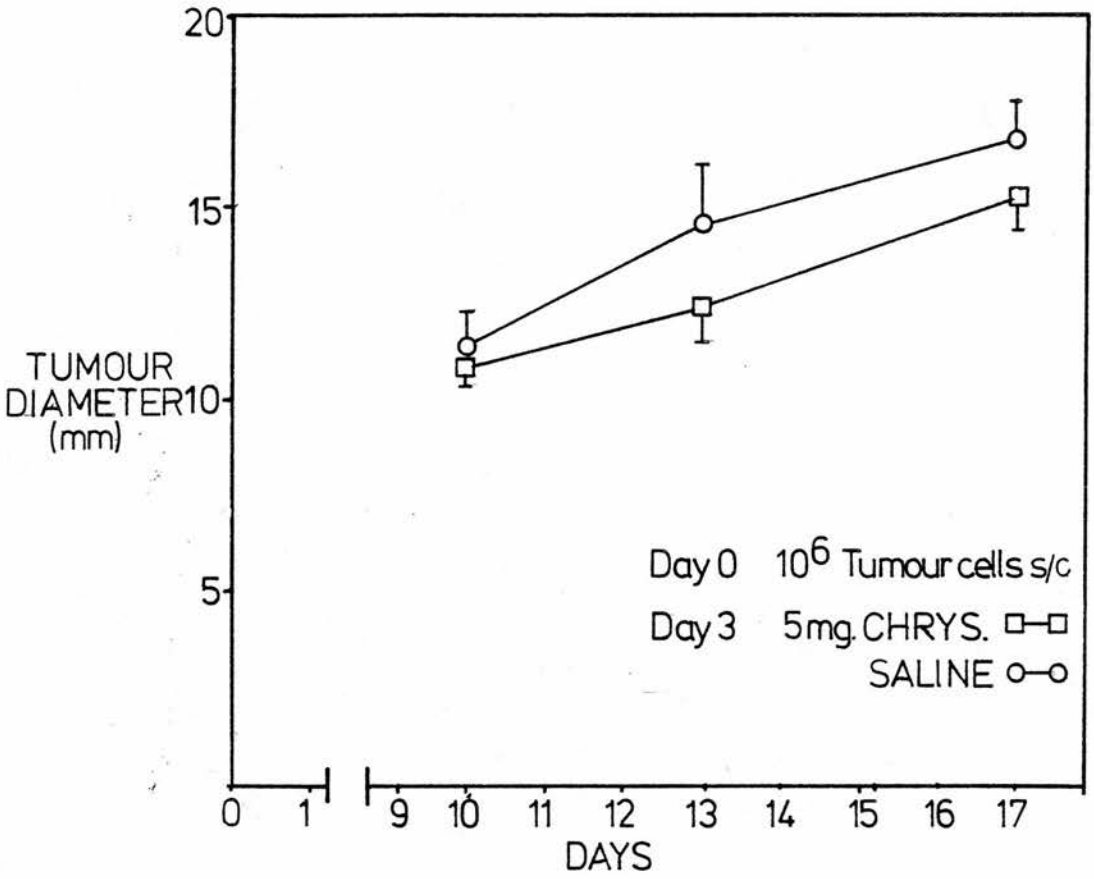


FIGURE 3.24 **Effect of intraperitoneal chrysotile on subcutaneous tumour growth. Significant differences between saline and chrysotile injected - Day 13 $P<0.01$; Day 17 $P<0.001$.**
Symbols denote $\bar{x} \pm$ one standard deviation (10 mice).

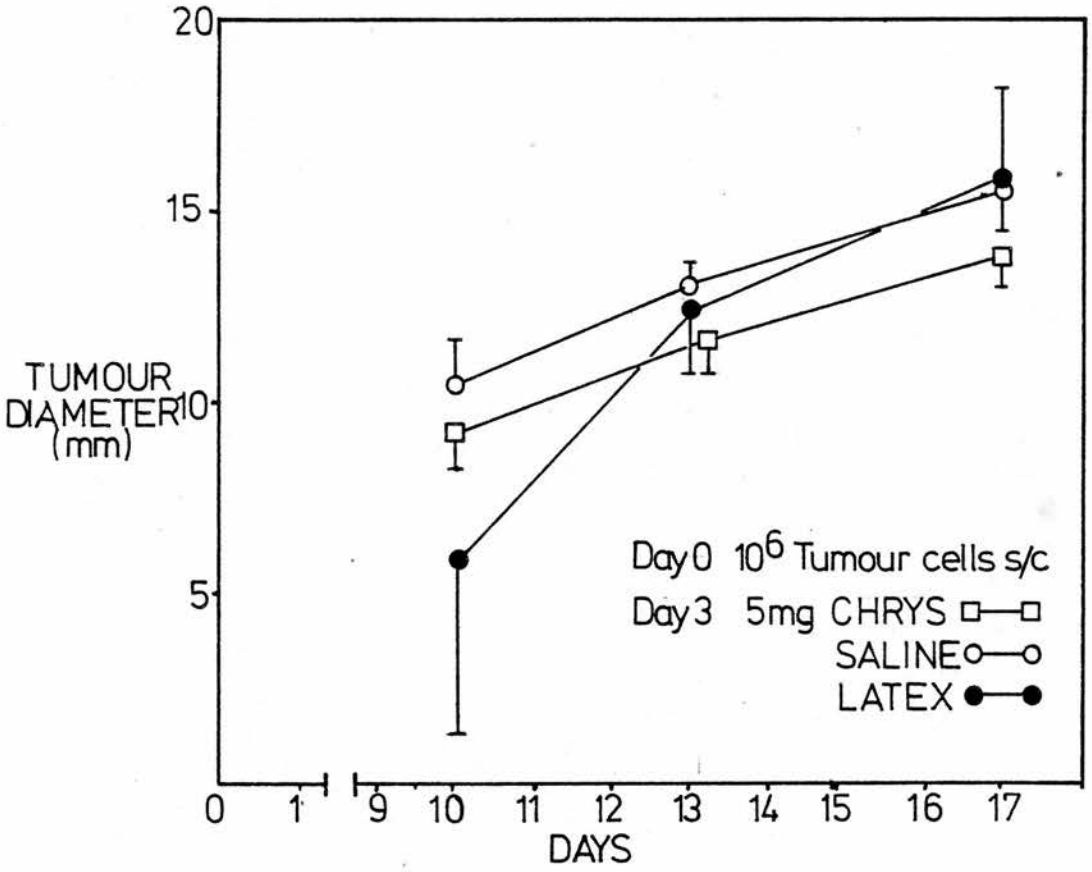


FIGURE 3.25 Effect of intraperitoneal chrysotile (5mg) and latex (1%) on subcutaneous tumour growth. Significant differences between saline and chrysotile - Day 10 $P<0.02$; Day 13 and Day 17 $P<0.002$. Significant difference between saline and latex - Day 10 $P<0.01$. Symbols denote $\bar{x} \pm$ one standard deviation (10 mice).

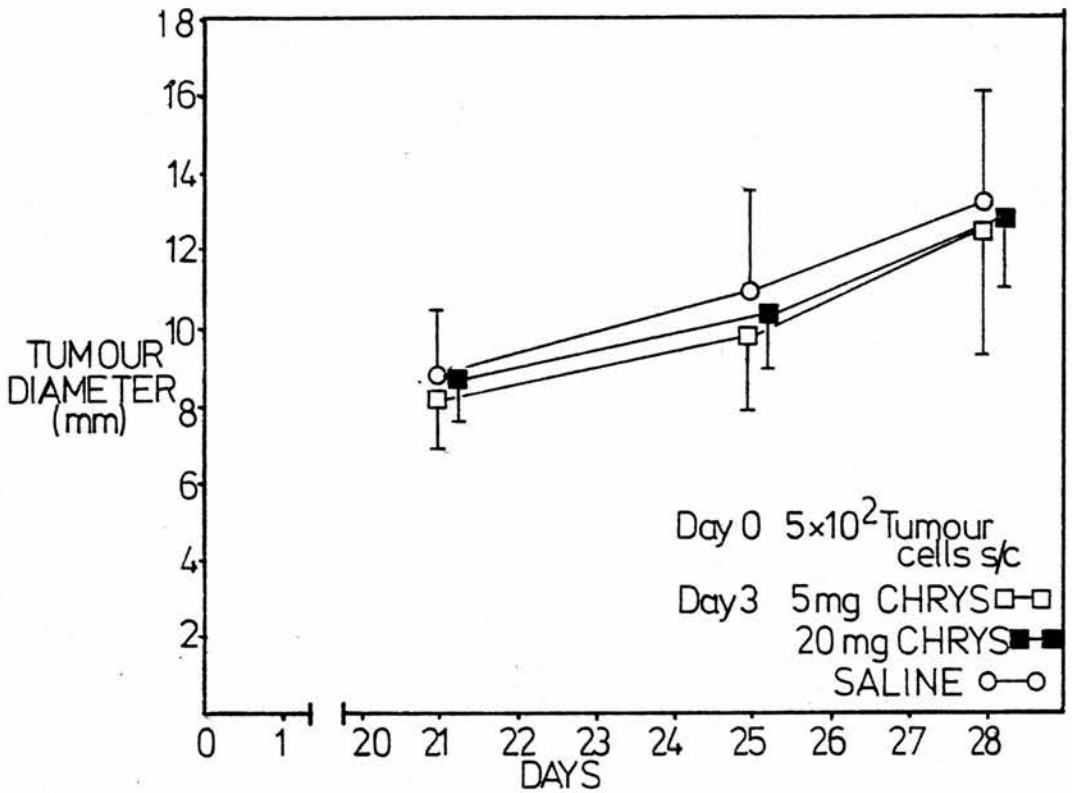


FIGURE 3.26 Effect of 5 mg and 20 mg chrysotile on tumours grown from a reduced tumour cell inoculation of $5x10^2$ cells. No significant differences. Symbols denote $\bar{x} \pm$ one standard deviation (10 mice).

points between chrysotile and saline injected animals.

(iii) 5 mg and 20 mg chrysotile 2 days prior to 5x10 s/c tumour cells

In further attempts to emphasise the effect of chrysotile obtained in 3.3.3.1(i) 5 and 20 mg of chrysotile were injected intraperitoneally 2 days before tumour cell inoculation with $5x10^2$ cells. Fig. 3.27 shows, by comparison with Fig. 3.28, that this protocol greatly enhanced the growth inhibitory effect of C. parvum injection which caused significantly (Day 28, 32 $P < 0.001$) smaller tumours than controls. Both 5 and 20 mg of chrysotile in this protocol, however, had no significant effect on tumour growth.

(iv) 5 mg crocidolite 3 days after 10^6 s/c tumour cells

To determine whether crocidolite, in the same protocol, had the same inhibitory effect as chrysotile on s/c tumour growth, 5 mg crocidolite was injected s/c 3 days after 10^6 s/c tumour cells (Fig. 3.28). C. parvum was also injected and caused significantly (Day 10 $P < 0.01$; Day 13 $P < 0.05$; Day 17 $P < 0.001$) smaller tumours but 5 mg crocidolite had no significant effect on tumour growth.

(v) Effect of quartz on tumour growth

Quartz given intraperitoneally as 20 mg 3 days prior to inoculation with 10^6 s/c tumour cells had no effect on tumour size except for a significant ($P < 0.02$) increase in tumour size on Day 20 (Fig. 3.29). Once again 20 mg of chrysotile had no significant effect on tumour growth rate.

3.3.4 Winn type assays using asbestos induced PEC

Three day chrysotile induced PEC were used at ratios of 10:1 and 100:1 in Winn assays of subcutaneous tumour growth. Fig. 3.30 shows that at both ratios of PEC:tumour cells there was no significant effect of chrysotile induced PEC compared to either CCH_1 cells alone or CCH_1

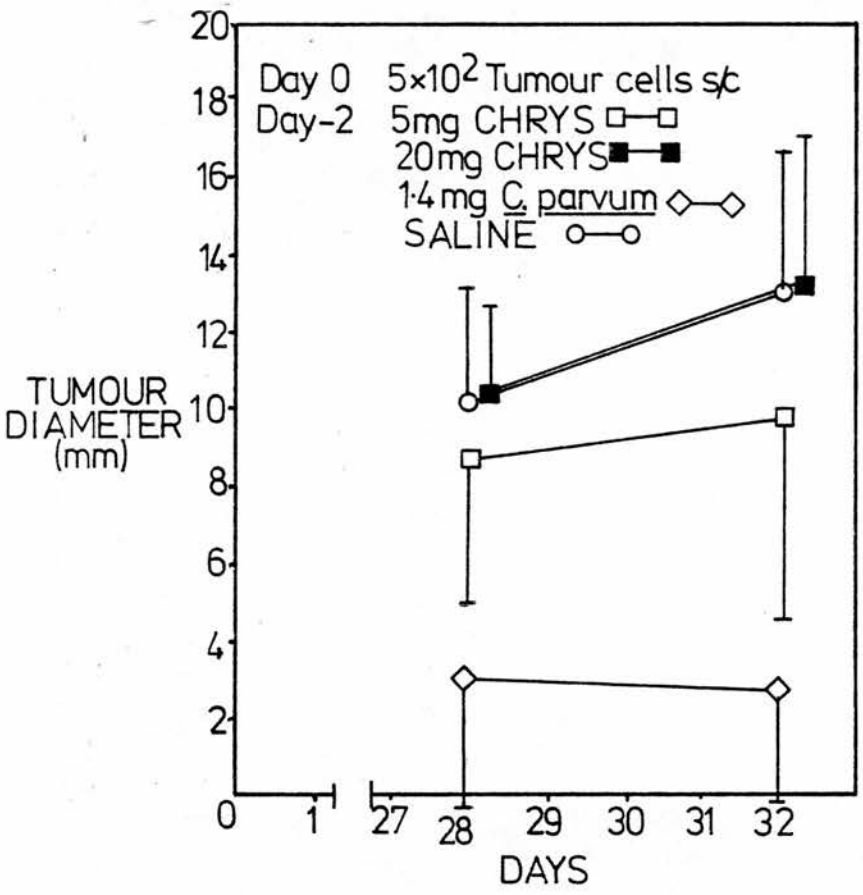


FIGURE 3.27 Effect of 5 and 20 mg chrysotile injected intraperitoneally 2 days before subcutaneous inoculation with the low dose ($5x10^2$) of tumour cells. No significant effects of chrysotile in this regime. C. parvum included as a positive control caused marked significant reductions in tumour size on days 28 and 32 compared to saline ($P<0.001$). Symbols denote $\bar{x} \pm$ one standard deviation (10 mice).

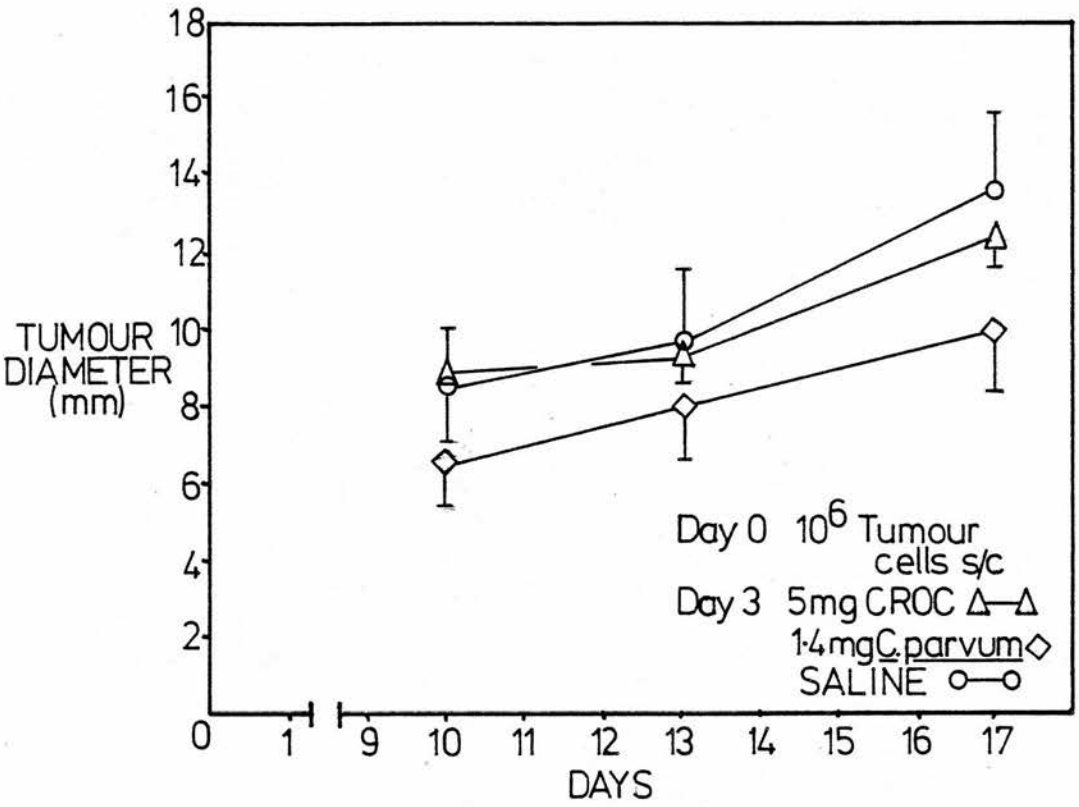


FIGURE 3.28 Effect of crocidolite (5mg) on subcutaneous tumour growth. No significant difference in tumour size between saline or crocidolite injected mice. C. parvum included as positive control produced significantly smaller tumours on Day 10 $P<0.01$; Day 13 $P<0.05$; Day 17 $P<0.001$.
Symbols denote $\bar{x} \pm$ one standard deviation (10 mice).

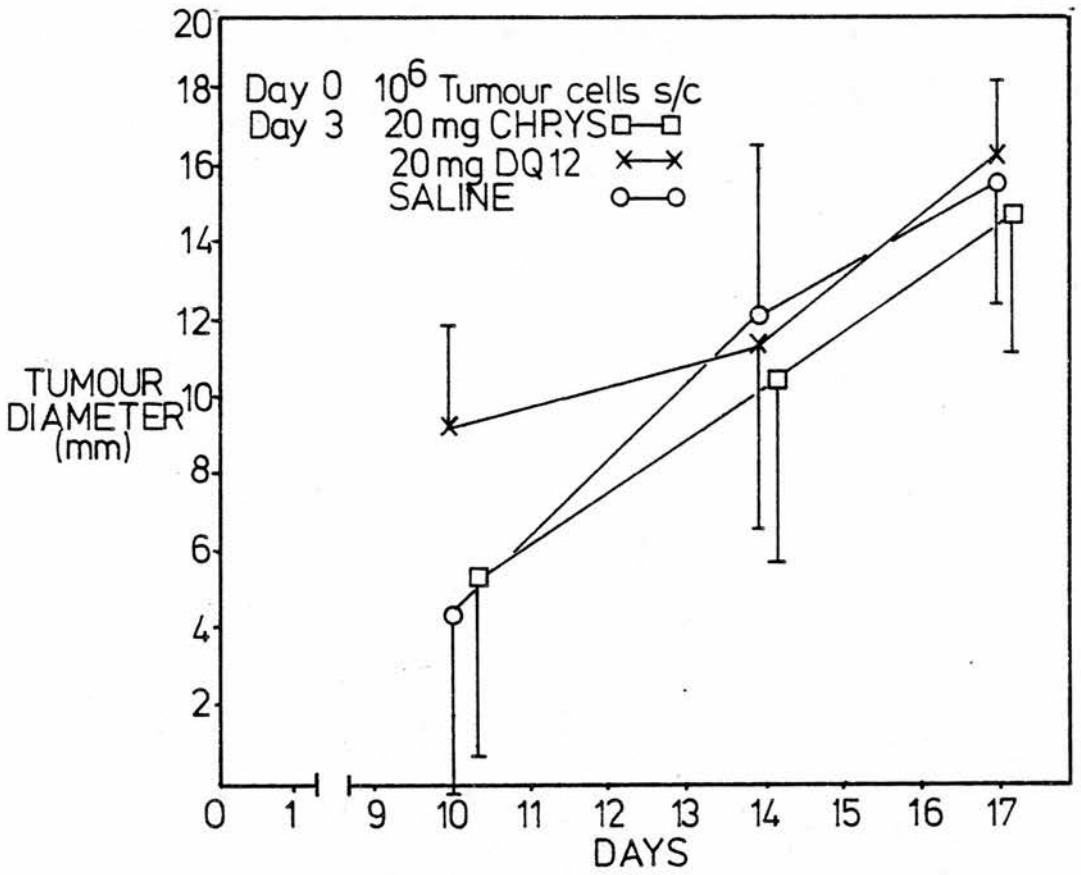
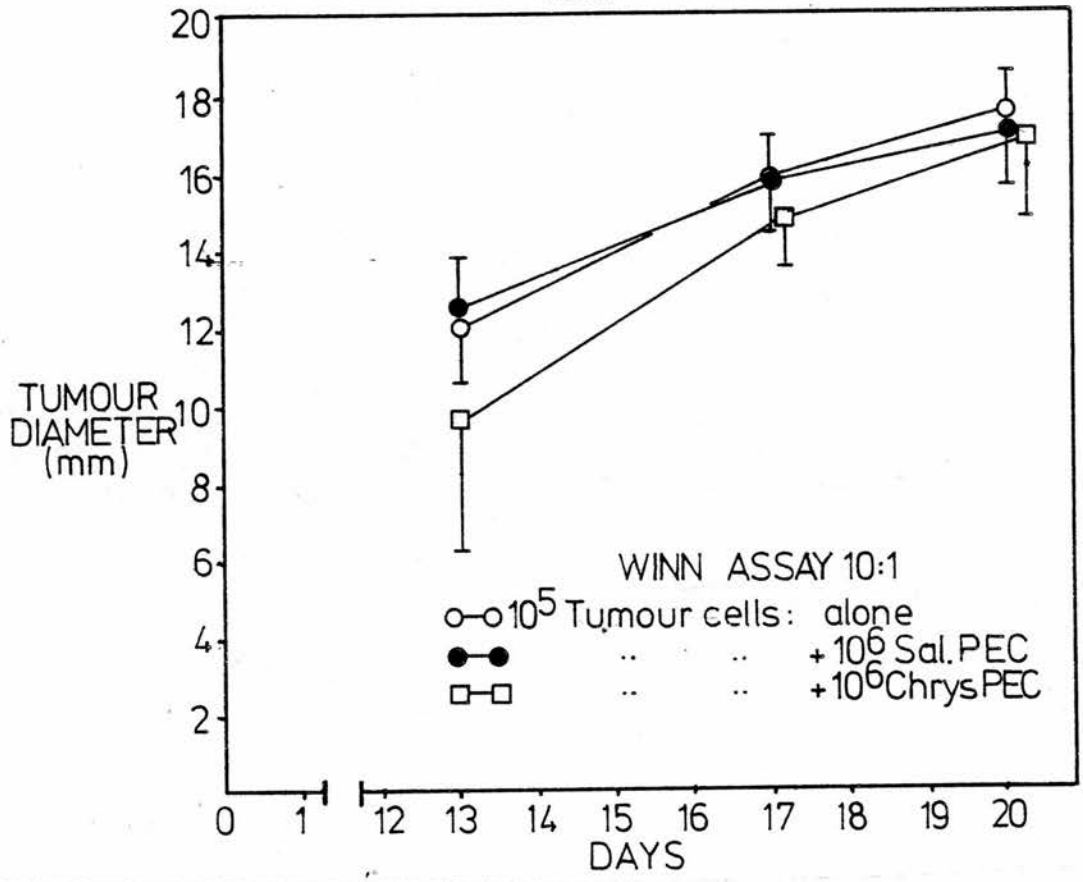


FIGURE 3.29 Effect of chrysotile (20 mg) and quartz (20 mg) on subcutaneous tumour growth. No significant effect of chrysotile injection compared to saline; significantly greater tumour diameter in quartz injected compared to saline injected mice on day 10 ($P<0.02$) ($\bar{x} \pm sd$: 10 mice)



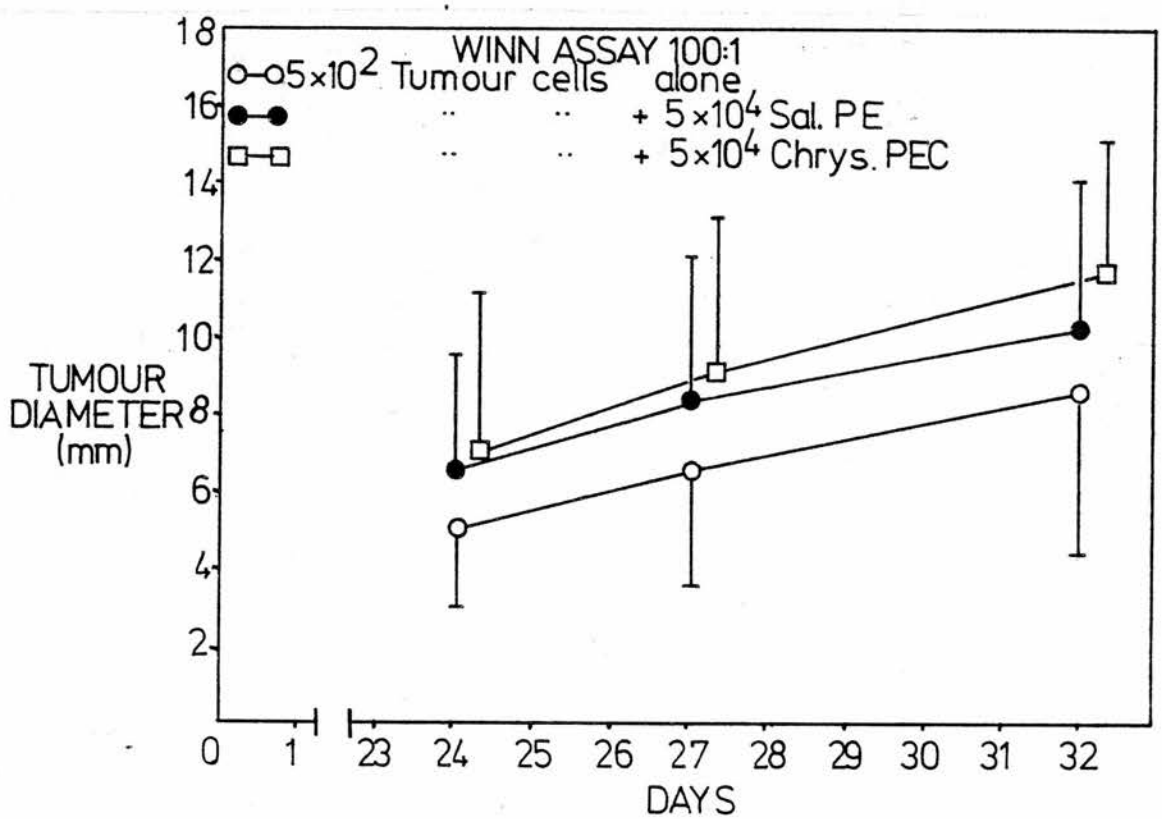


FIGURE 3.30 Effect of chrysotile PEC in Winn assays with tumour cells at ratios of 10:1 (upper) and 100:1 (lower). Controls comprise saline PEC:tumour cells at the same ratio and tumour cells only. Significantly smaller tumour diameter found only with chrysotile PEC at 10:1 ratio on day 13 ($P < 0.02$).
Symbols denote $\bar{x} \pm$ one standard error (10 mice).

cells mixed with saline induced PEC at the same ratios except for 10:1 day 13 where chrysotile PEC caused a significantly ($P < 0.02$) smaller tumour size compared to saline PEC; this difference had disappeared by day 17 and 20.

3.3.5 Effect on tumour growth of mixing various amounts of crocidolite with the inoculating tumour cells

In an effort to produce local macrophage activation at the tumour inoculation site, it was decided to mix various doses of asbestos with the inoculating tumour cells and measure the effect on tumour growth. Crocidolite was chosen rather than chrysotile since it has a less active surface and would be less likely to cause direct toxic effects on the tumour cell membrane; crocidolite was incubated for an hour at 37°C in cRPMI to coat the fibre and further reduce any direct toxic effects. Fig. 3.31 shows a dose dependent retardation of tumour growth with crocidolite added to the inoculating tumour cells. Significant ($P < 0.05$ to $P < 0.001$ see Fig. 3.31 for details) differences, compared to tumour cells only, were produced by 50 and 500 µg crocidolite at all 3 time points.

3.3.6 Effects of asbestos and asbestos leachate on growth of CCH_1 tumour cells in vitro

3.3.6.1 Asbestos

Fig. 3.32 shows the pooled results of 4 separate experiments to measure the direct effects of asbestos on tumour cell tritiated thymidine uptake in vitro. It is evident that there is no stimulatory effect of either crocidolite or chrysotile at low doses. Over the first 3 orders of magnitude there is relatively little effect (up to 10 µg/well) with crocidolite being slightly more inhibitory than chrysotile. At 100 µg/well, a highly unrealistic dose, the results become erratic with

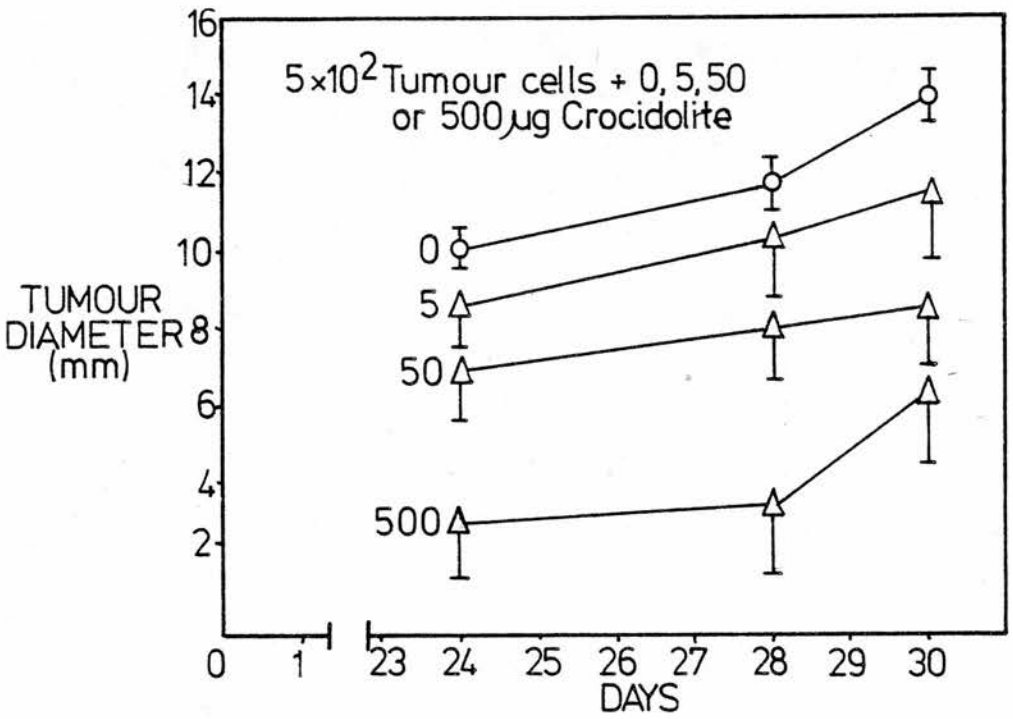


FIGURE 3.31 Effect on tumour growth of mixing 5, 50 or 500 µg of crocidolite with $5x10^2$ inoculating tumour cells. Significantly smaller tumours produced by 50 and 500 µg of crocidolite compared to no crocidolite on - Day 24 50 µg $P<0.05$; 500 µg $P<0.001$. Day 28 50 µg $P<0.05$; 500 µg $P<0.001$. Day 30 50 µg $P<0.02$; 500 µg $P<0.01$.
Symbols denote $\bar{x} \pm$ one standard error (10 mice).

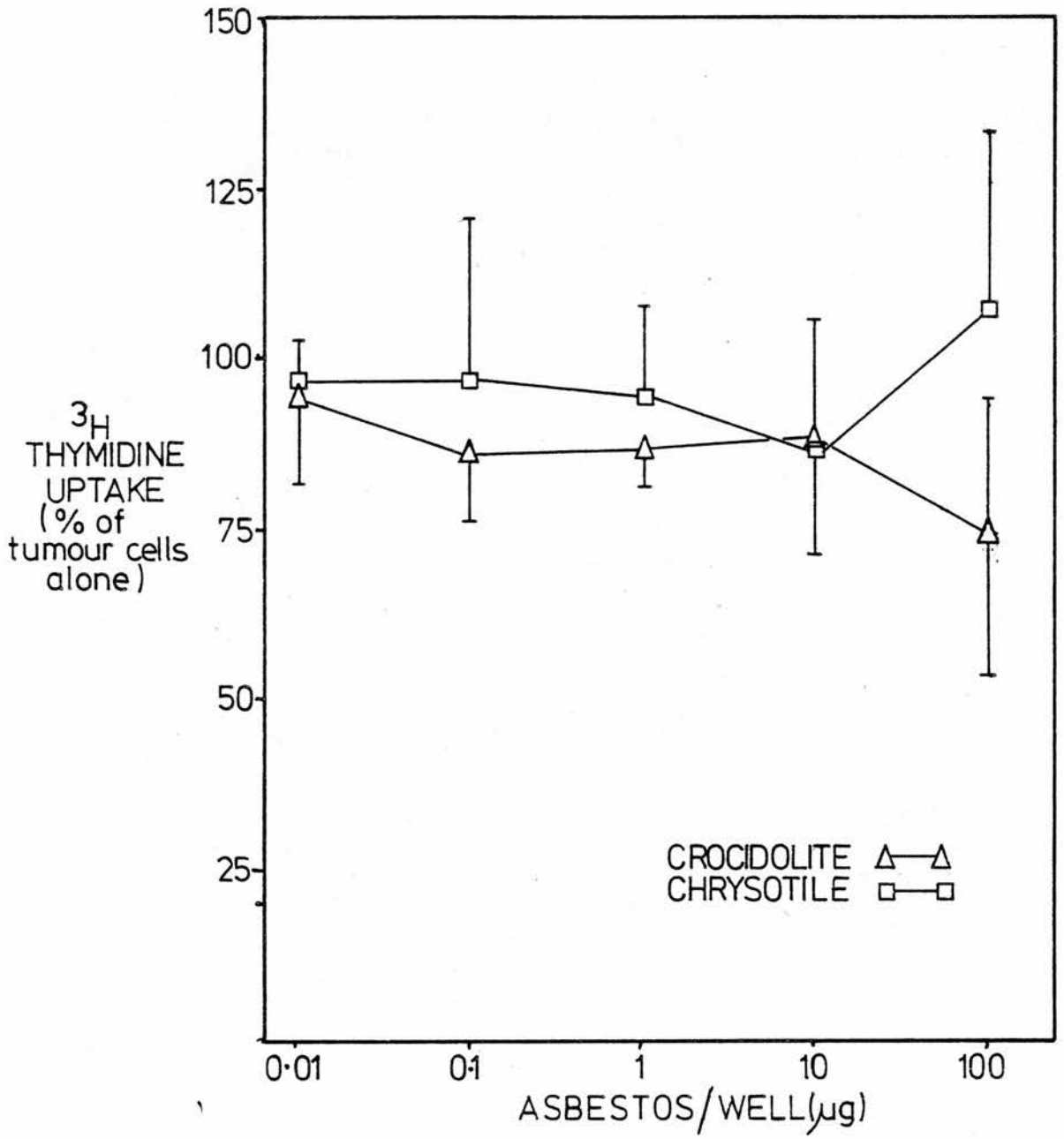


FIGURE 3.32 **Effect of 0.01-100 µg/ well of asbestos on uptake of thymidine by CCH_1 tumour cells.**
Symbols denote $\bar{x} \pm$ one standard deviation.
(data from 3 separate exp^s)

crocidolite inhibiting thymidine uptake and chrysotile apparently stimulating uptake. Fig. 3.33 shows that the effect of asbestos was considerably less than that produced by inert latex particles given over 5 orders of magnitude when latex produced a clear dose dependent inhibition of thymidine uptake.

3.3.6.2 Asbestos leachate

Fig. 3.34 shows that there was no obvious dose dependent effect of medium in which asbestos had been incubated (leachate) on tumour cell proliferation with maximum inhibition of between 5 and 20% being present between 0.5 and 50% of leachate by volume.

3.3.7 Effect on tumour growth of having cultured the inoculating tumour cells in vitro in the presence of crocidolite

In order to test whether incubation with crocidolite, prior to injection into mice, could alter the tumourigenicity of the CCH_1 cells they were cultured with 2.5 µg of crocodolite (pre-coated by incubation with cRPMI) for 2 days then harvested and inoculated subcutaneously in mice. Tumour growth was then monitored and as Fig. 3.35 shows, pre-incubation of CCH_1 cells with crocidolite had no significant effect on their ability to form tumours compared with tumour cells grown in parallel culture in the absence of crocidolite.

3.4 The use of Concanavalin A to study the effect of asbestos on macrophage membranes

3.4.1 Visualisation of Con A receptors during interactions between macrophages and asbestos

3.4.1.1 Light microscopy

Macrophages were treated with chrysotile asbestos for up to 1 hour in serum or serum free conditions followed by demonstration of Con A

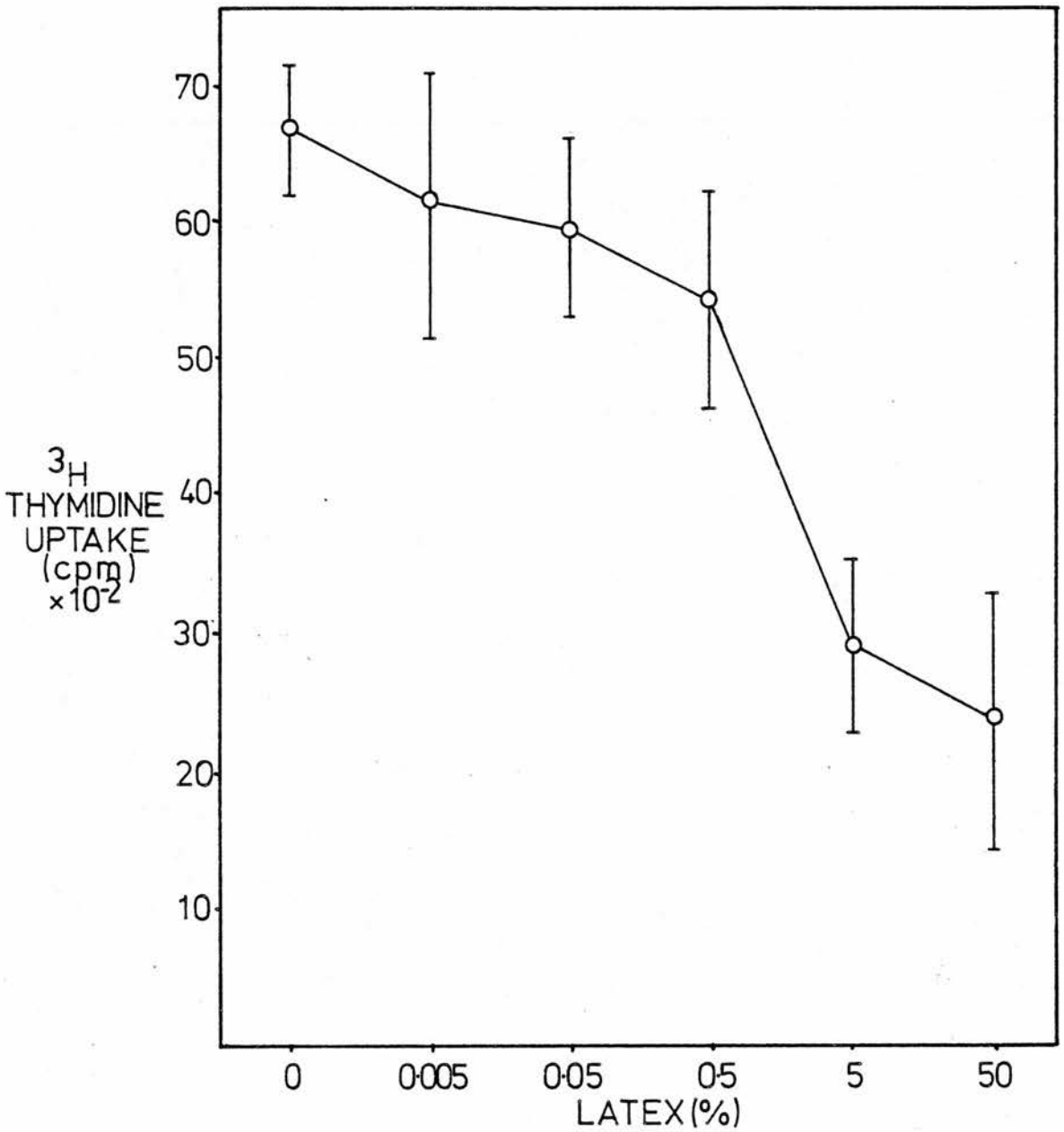


FIGURE 3.33 **Effect of increasing doses of latex on uptake of tritiated thymidine by CCH_1 tumour cells.**
Symbols denote $\bar{x} \pm$ one standard deviation (triplicate wells : repr exp.)

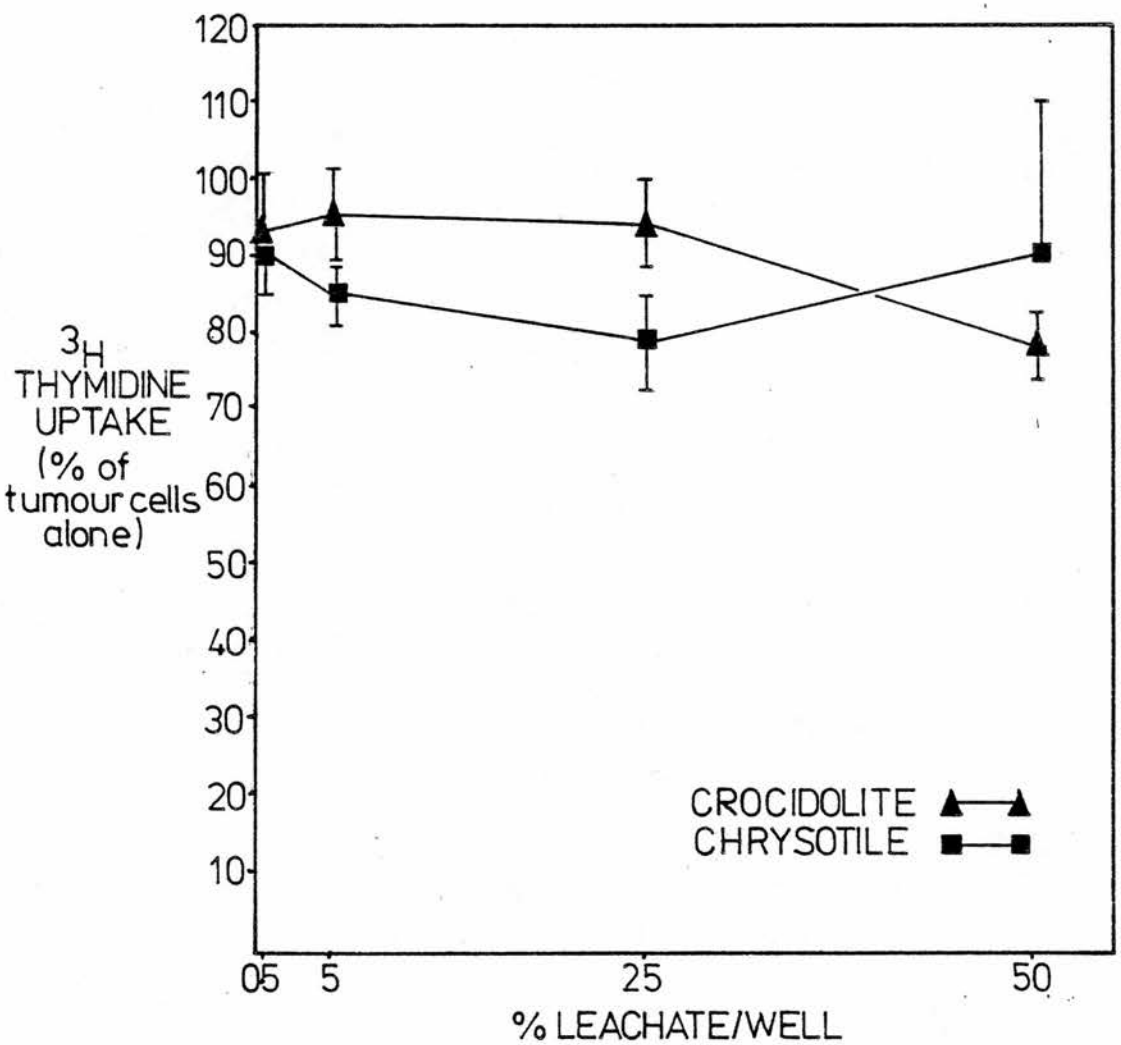


FIGURE 3.34 **Effect of asbestos leachate on uptake of tritiated thymidine by CCH_1 tumour cells.**
Symbols denote $\bar{x} \pm$ one standard deviation.
pooled data from 3 separate exp^{s}

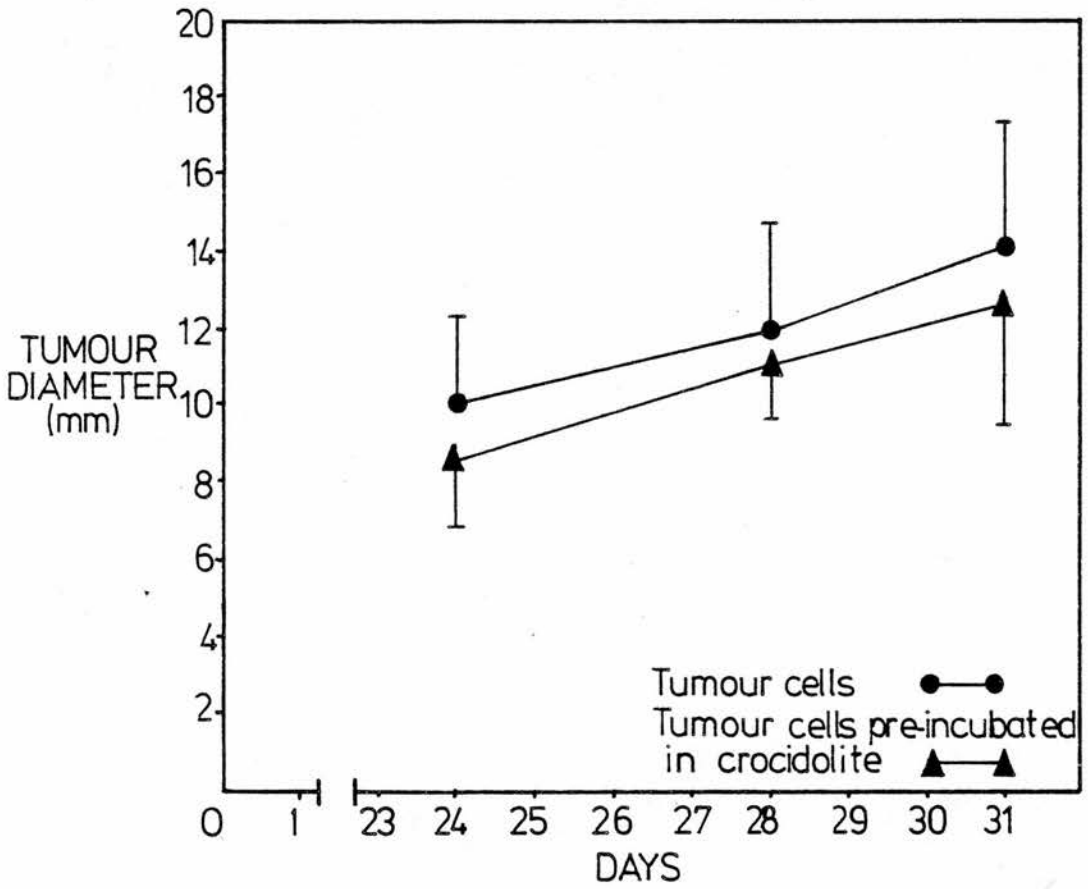


FIGURE 3.35 **Effect on tumour growth of having pre-incubated the inoculating tumour cells in crocidolite for 2 days. Symbols denote $\bar{x} \pm$ one standard deviation** (10 mice).

receptors by FITC-ConA staining. Any changes in the disposition of the Con A receptors in the membrane, following contact with asbestos, would therefore be revealed. As described below only a few instances were observed, where there was obvious perturbation to Con A staining.

Fig. 3.36 shows bright field and fluorescence illuminated exposures of the same cells 15 minutes after the addition of chrysotile in serum free conditions. The arrow indicates an area of bright fluorescence at a point where a chrysotile fibre is being phagocytosed, or is in contact with the macrophage membrane. Due to the curve in the fibre, the fibre is making contact with the cell in a plane perpendicular to the plane of the photograph as was discernible by focusing. Fig. 3.37 shows cells treated in the same way as those in Fig. 3.36; a straight line of fluorescence is seen to be crossing one of the cells (arrowed) and this coincides with a chrysotile fibre lying across the cell at the same angle. Unfortunately the fibre in question is partly obscured in the bright field exposure by a large defocussed fibre passing in a plane well above that of the cell due to its curliness. Fig. 3.38 shows cells 30 minutes after addition of chrysotile in serum containing medium and shows a macrophage apparently trying to engulf a long fibre (arrow). At the point where the membrane closes around the fibre like a sleeve, at its furthest extremity from the cell body, there is a slight but distinct increase in fluorescence intensity. Fig. 3.39 shows cells 30 minutes after the addition of chrysotile in the presence of serum and a macrophage (arrow) ingesting a small chrysotile fibre is seen to have a concentration of fluorescence around the point of phagocytosis at the membrane.

The number of cells showing some perturbation of the Con A staining, at points of membrane/chrysotile interaction were few, representing less than 1% at all time points. It was however impossible to tell whether

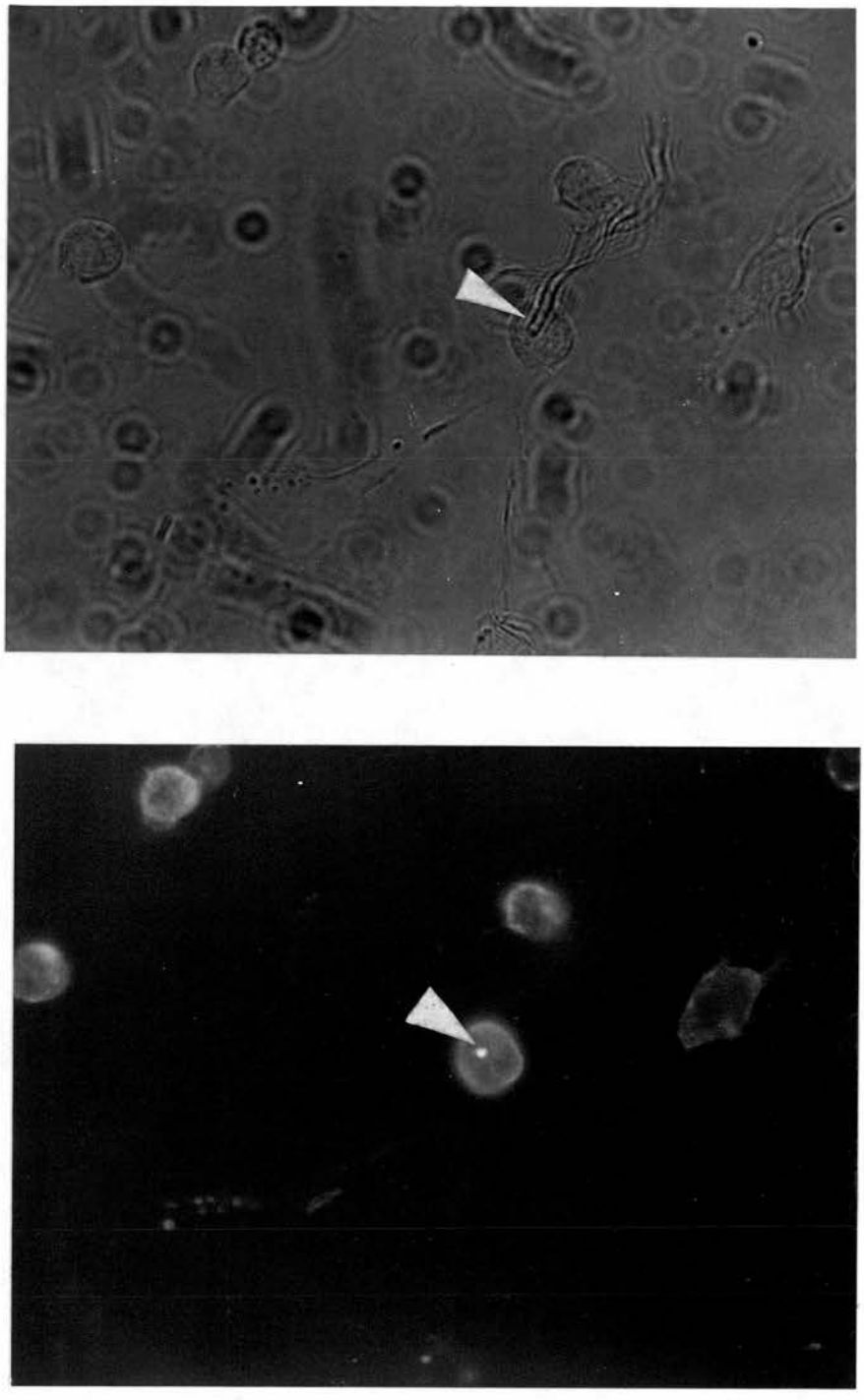


FIGURE 3.36 Macrophages incubated with chrysotile asbestos then stained with FITC-Con A. Upper photograph taken under bright field illumination; lower under fluorescence illumination. Arrow shows area of bright fluorescence where fibre is in contact with a cell in a plane perpendicular to the paper. Mag. x 600

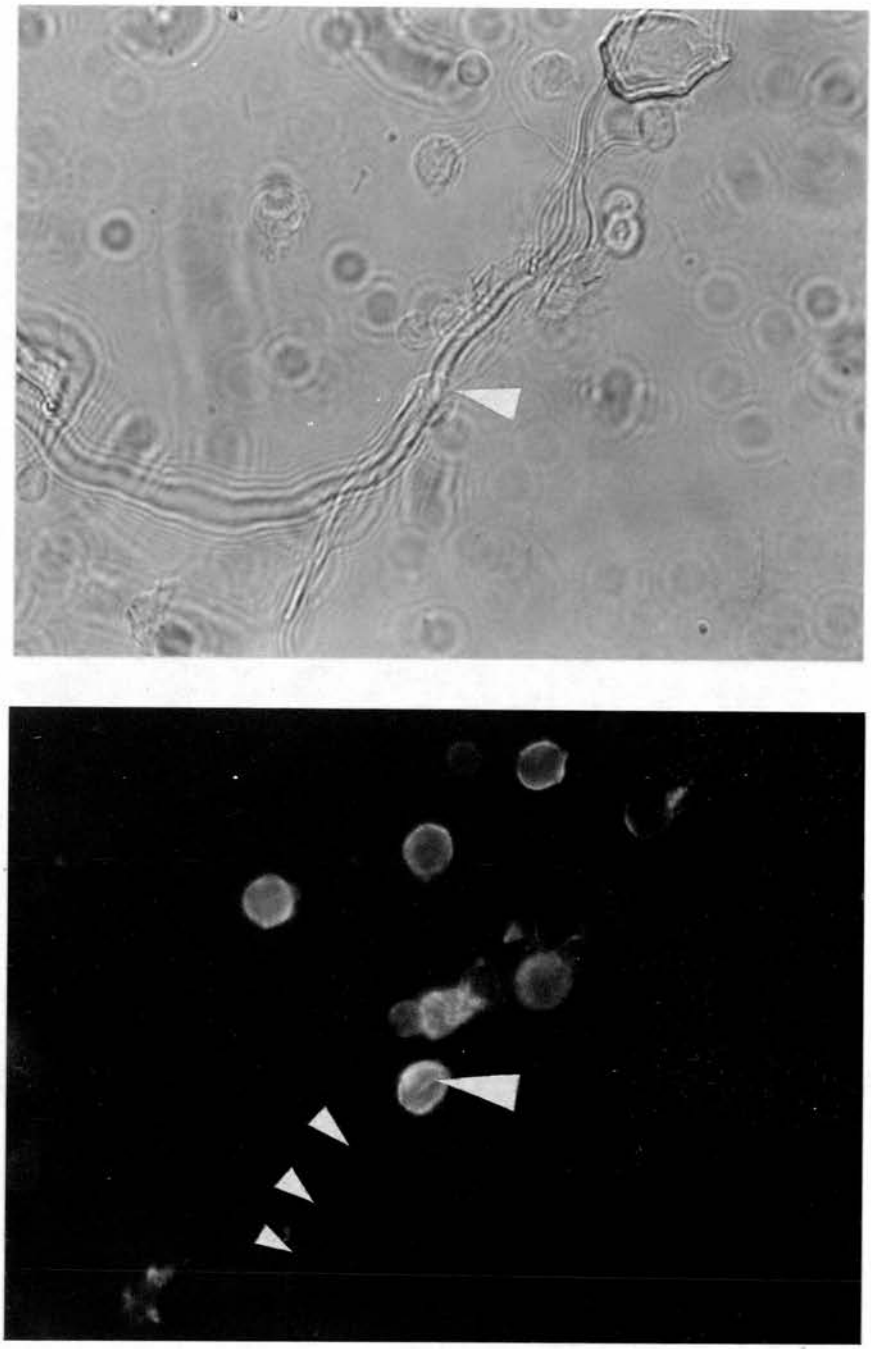


FIGURE 3.37 Macrophages prepared as in Fig. 3.36. Arrow in lower indicated a straight line of fluorescence which coincides with a fibre lying across a macrophage but which is not clearly seen in the bright field (upper) view due to the defocussed fibre lying above it. In the lower figure the very faint outline of the fibre is seen continuing on from the line of fluorescence (arrows).

Mag. x 600

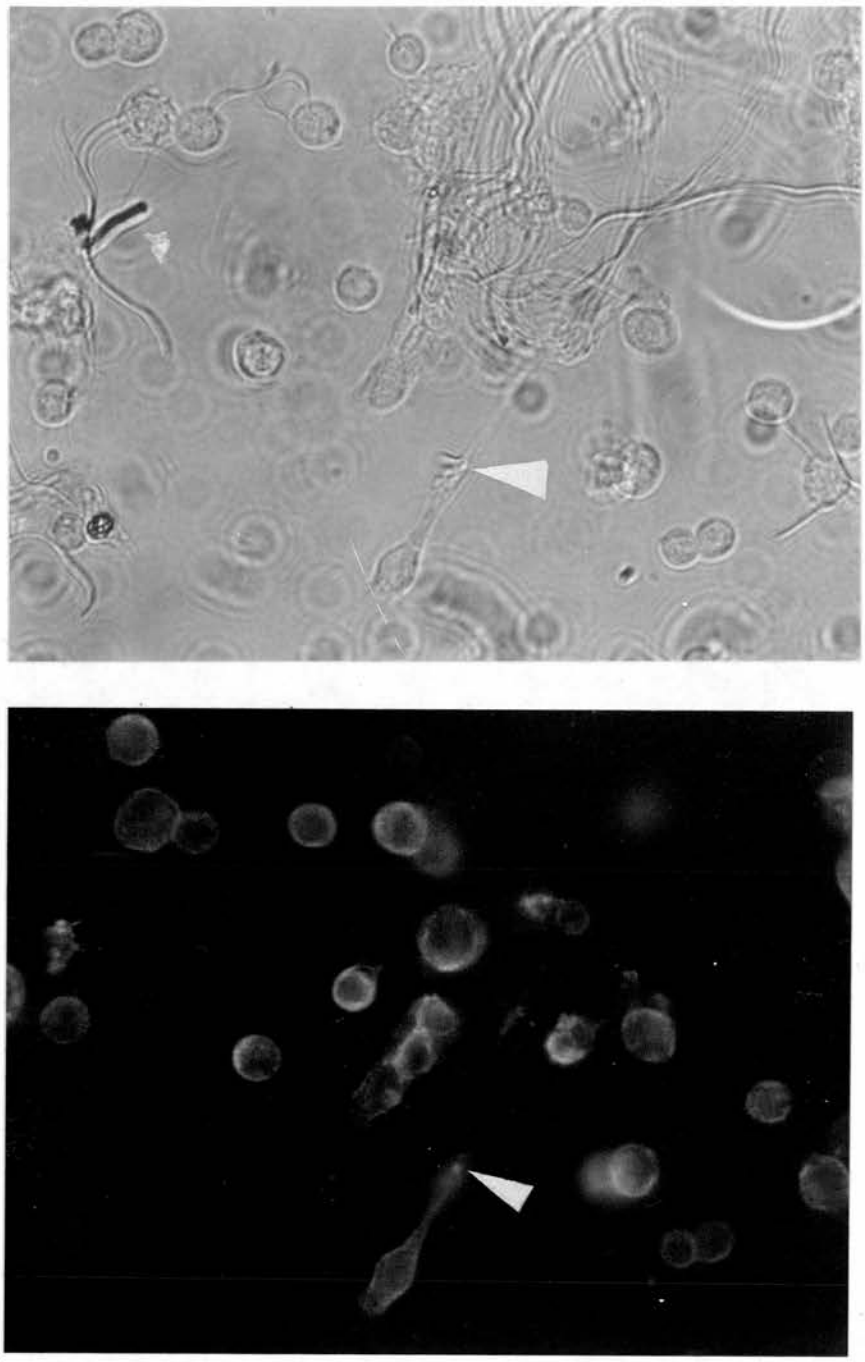


FIGURE 3.38 Macrophages prepared as in Fig. 3.36; arrow indicates macrophage spread out and ingesting a fibre. At the point where the membrane closes around the fibre there is a concentration of fluorescence.

Mag. x 600

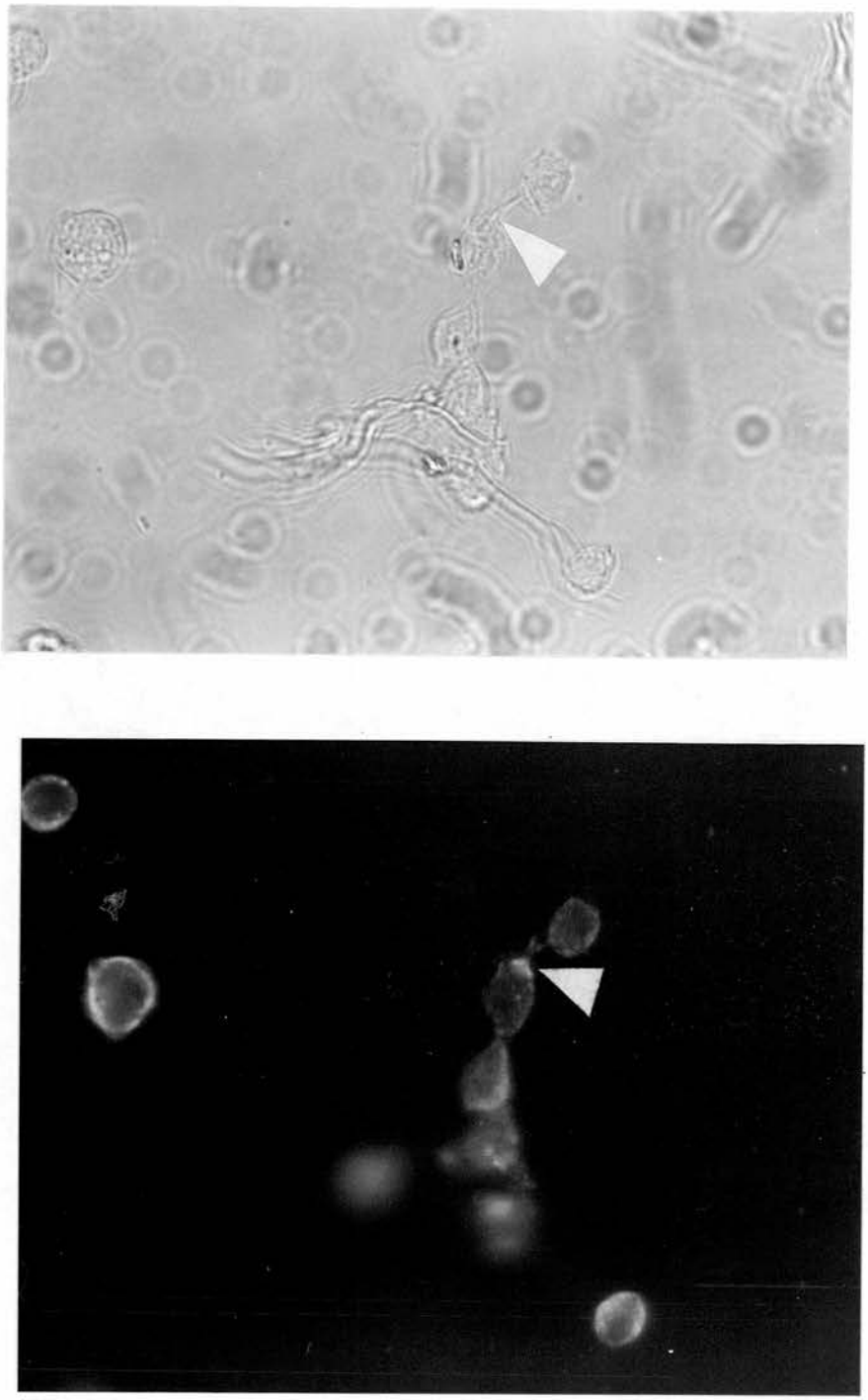


FIGURE 3.39 Macrophages prepared as in Fig. 3.36; arrow shows concentration of fluorescence at membrane where fibre enters the cell.

Mag. x 600

some "concentrations" of fluorescence at the membrane, which were apparently not asbestos associated, were in fact due to interactions of the membrane with chrysotile fibres below the resolution of the microscope. It follows from this, also, that the perturbation in Con A distribution shown here, represents those produced by the larger, resolvable fibres.

Concentrations of fluorescence were present at points of contact between macrophage membrane and chrysotile fibres in both non-serum and serum containing conditions. The aggregation of fluorescence at points of contact between asbestos fibres and macrophage membranes was always present along with some "ring" staining of the sort obtained with normal macrophages. The areas of increased fluorescence intensity were not, therefore, caps; caps are usually present as aggregates of fluorescence with the normal "ring" fluorescence absent since all the receptors have been drawn into the cap. The difference between a cap and increased intensity of fluorescence at any point is apparent by comparing Fig. 3.36 with Fig. 3.45.

Occasionally Con A positive "beads" were seen on fibres (e.g. Fig. 3.36); the exact nature of these was unclear although they could represent organic material in the UICC sample or regions of adsorption of FITC-Con A on to the fibre surface.

3.4.1.2 Electron microscopy

As shown in Fig. 3.40 the ultrahistochemical demonstration of macrophage surface bound Con A was successful with the black reaction product confined to the plasma membrane and a few vesicles near the membrane probably representing infoldings of the membrane caught in section. Fig. 3.40 also reveals Con A to be bound only to the free surface of a macrophage which has been spread on glass although it is not possible to ascertain whether the lack of reaction product on the adherent

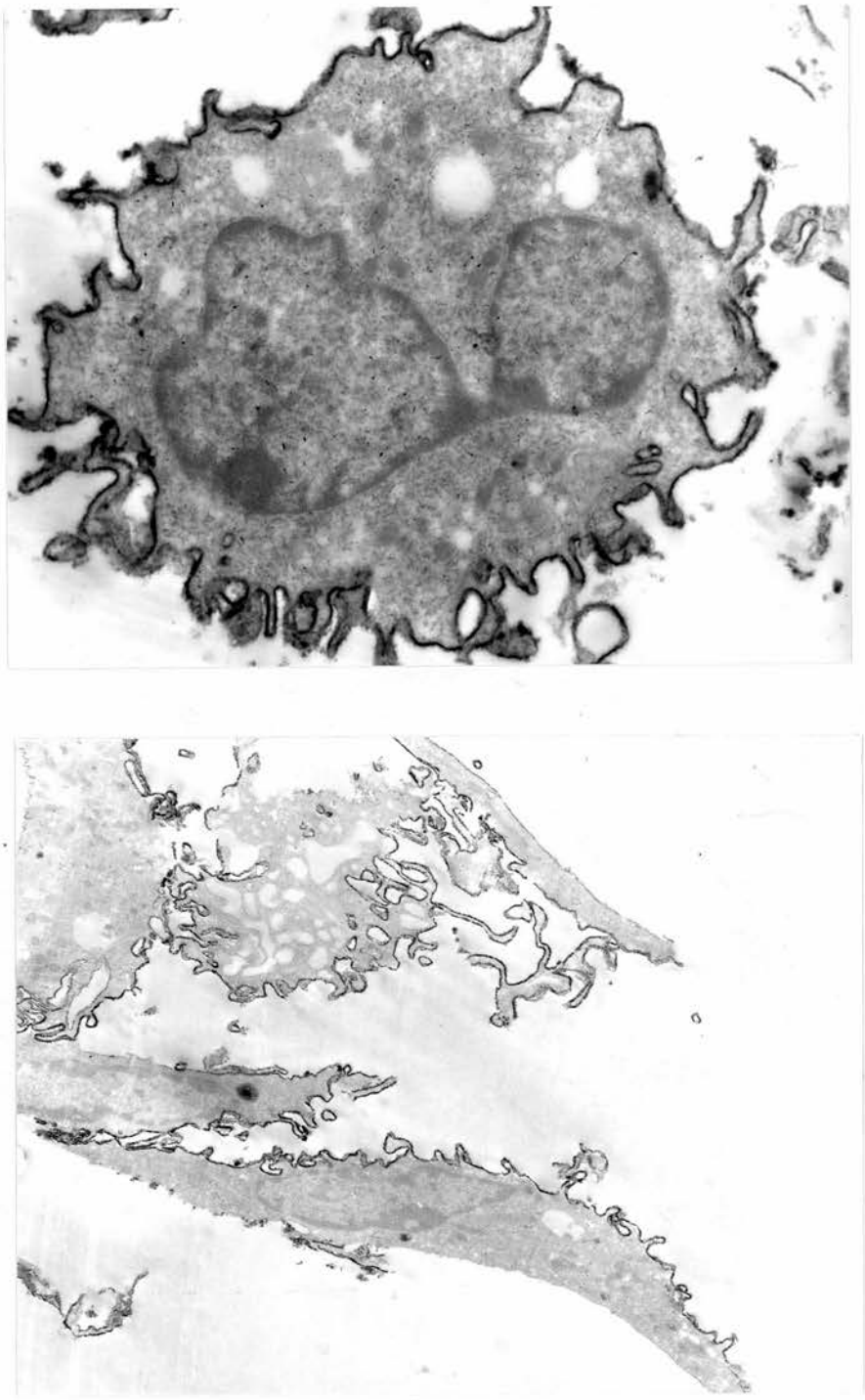


FIGURE 3.40 Macrophages stained with peroxidase to reveal Con A bound to the surface.
Upper - high power view to show that reaction product is confined to the surface.
Lower - low power view revealing the majority of reaction product to be confined to the free surface of the macrophages.
Magnification - upper x 7,500;
lower x 3,000.

aspect was due to trauma to the adherent surface during removal of the cells from the petri dish.

Close interactions between macrophage membrane and asbestos were present at 30 and 60 minutes after addition of chrysotile to the cultures. Fig. 3.41 (upper) shows an area of membrane where a bundle of fibres is in contact with macrophage membrane in serum containing conditions; the distribution of reaction product at the membrane can be seen to be unaltered by the interaction. As shown in Fig. 3.41 (lower) at sites of interaction between chrysotile and macrophage pseudopodal extensions there is no evidence of obvious disturbance to the membrane distribution of Con A receptors. Fig. 3.42 upper and lower are higher power views and once again there is no obvious disturbance to the distribution of reaction product where the asbestos is in contact with the macrophage membrane. Figs. 3.43 upper and lower show phagosomes containing chrysotile; the density of the reaction product on the phagosomal membrane presumed newly derived from the plasma membrane at a site of interaction between fibre and membrane, is similar to that of the overlying plasma membrane.

These experiments showed that at no site of interaction between chrysotile and macrophage was there obvious evidence of clustering of Con A receptors either in serum containing or serum free conditions using this ultracytochemical demonstration of Con A receptors.

It should be emphasised that the ultracytochemical method used to demonstrate surface bound Con A was probably not capable of resolving small local variations in Con A density due to a combination of the coarseness of the reaction product and the thickness of the section necessitated by the difficulty of cutting blocks with so much chrysotile in them.

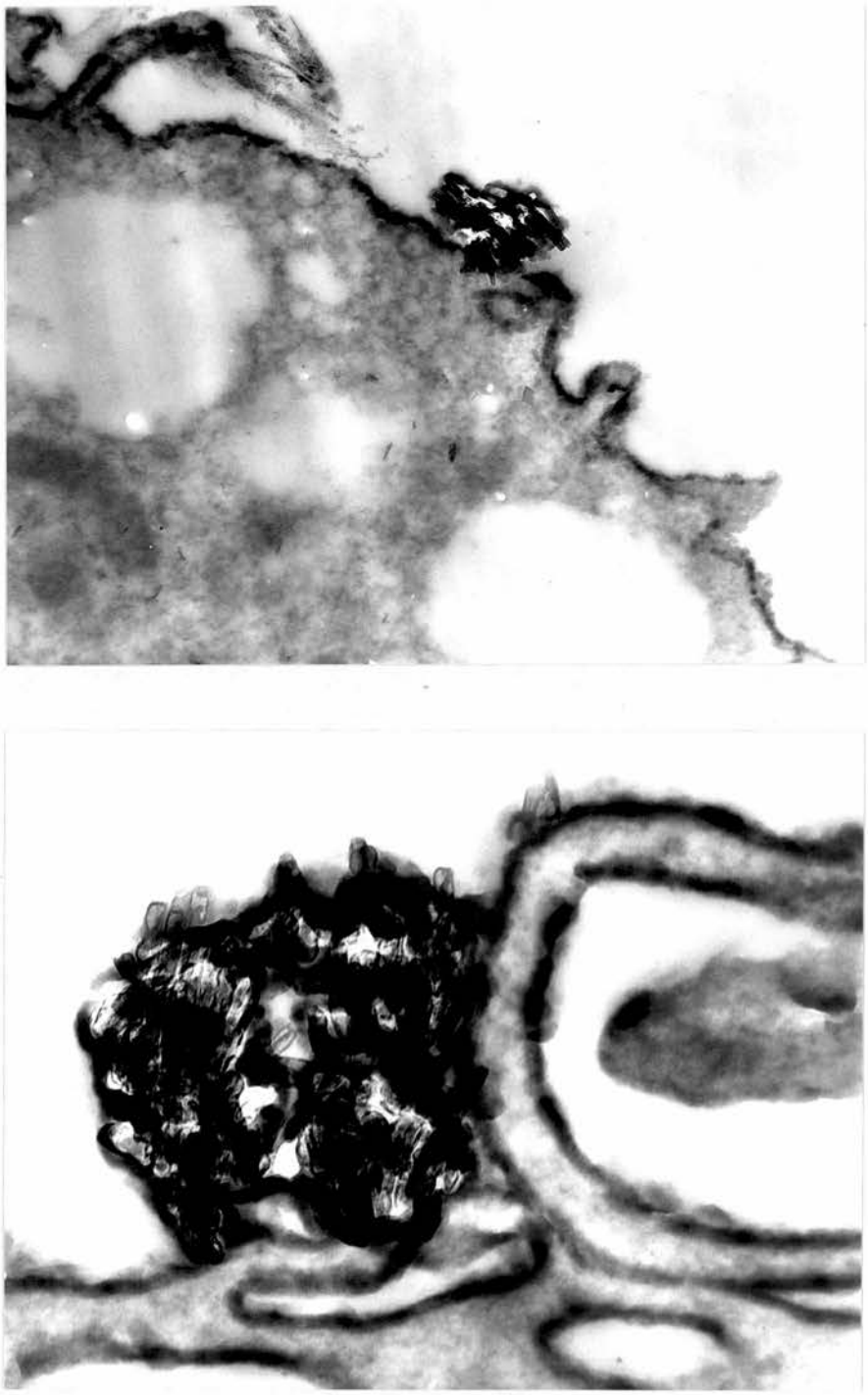


FIGURE 3.41 Macrophages prepared as in Fig. 3.40. In both the low (upper) and high (lower) power views shown there is no evidence of alterations in distribution of reaction product at sites of membrane chrysotile interaction.
Mag. - upper x 15,000; lower x 25,500.

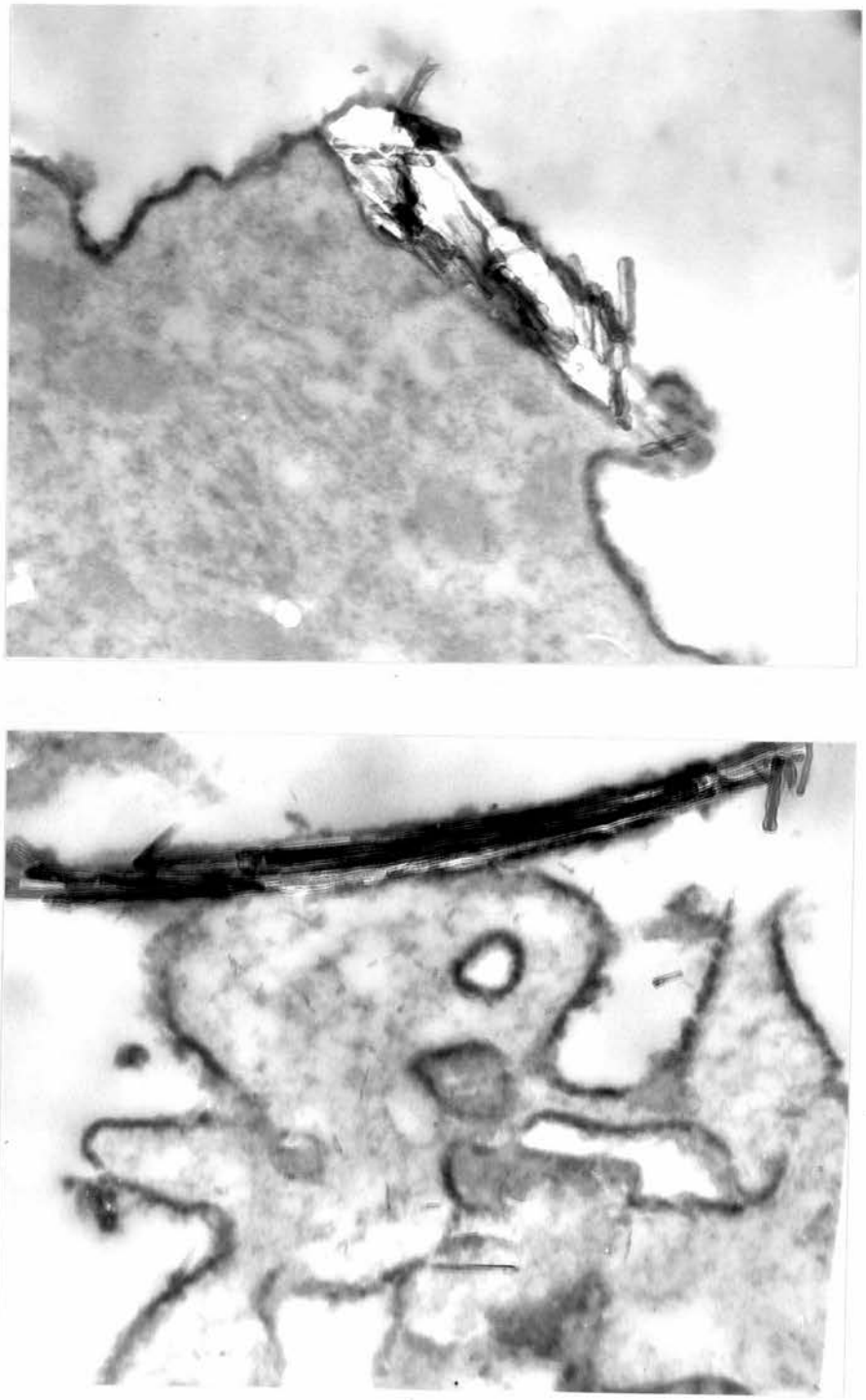


FIGURE 3.42 Macrophages prepared as in Fig. 3.40. Chrysotile in contact with macrophage membrane over an extensive area but failing to show any marked effect on reaction product distribution.
Mag. - upper x 20,000; lower x 20,000

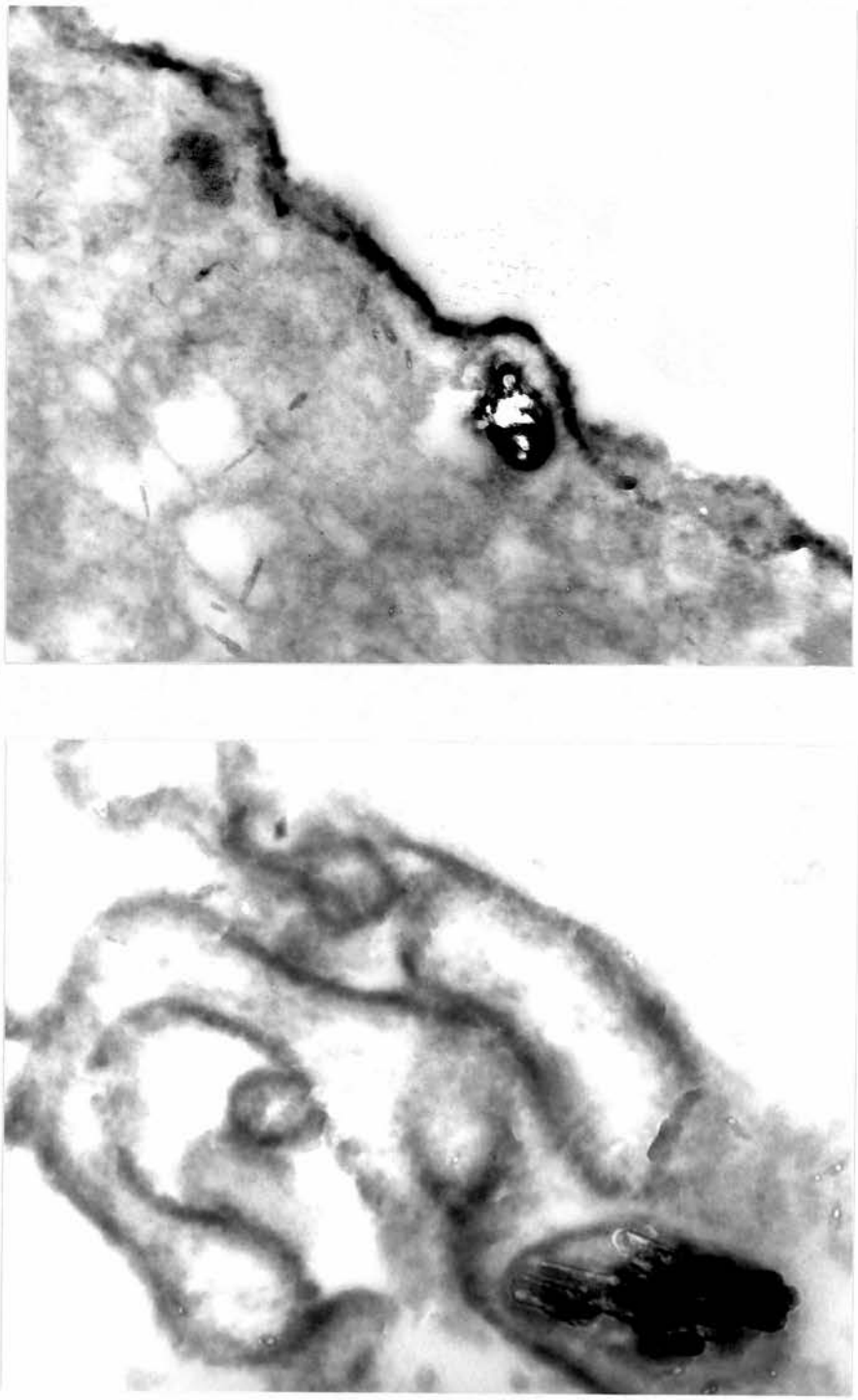


FIGURE 3.43 Macrophages prepared as in Fig. 3.40. Chrysotile present inside newly formed phagosomes close to the macrophage surfaces. No obvious differences in distribution of reaction product in phagosomes compared to the surface membrane from which they were derived. Mag. - upper x 15,000; lower x 25,500.

3.4.2 Concanavalin A induced capping in macrophages

3.4.2.1 Appearance of Con A caps in activated macrophages

Control 3 day saline induced peritoneal macrophages incubated at 37°C with FITC-Con A then fixed and viewed under fluorescent illumination had the appearance shown in Fig. 3.44 (upper); even "ring" fluorescence was present. Macrophages induced 3 days after chrysotile injection and treated in the same way, however, had a population comprising more than 30% approximately of cells which had bright "caps" of fluorescence and absence of ring staining as shown in Fig. 3.44 (lower). Fig. 3.45 shows both bright field and fluorescent views of a group of capped, chrysotile induced macrophages showing the caps to be present, in general, at one pole of the cell. The caps were similar to those described by Pick and Wilner (1979) in peritoneal macrophages.

3.4.2.2 Proportion of capping macrophages induced in the peritoneal cavity by different agents

As shown in Table 3.3 the proportion of macrophages which capped on incubation with Con A was, on average, below 5% for saline induced macrophages and also for macrophages induced by proteose peptone and latex. Injection of chrysotile, crocidolite or C. parvum however produced an increase in the percentage of capping macrophages. The proportion of caps in 3 day chrysotile, crocidolite and 5 day C. parvum was significantly ($P < 0.001$) greater than that of 3 day saline, proteose peptone or latex induced macrophages. The proportion of caps in 3 day chrysotile induced macrophages was significantly ($P < 0.02$) greater than that in 3 day crocidolite induced macrophages.

3.4.2.3 Time course of capping

Fig. 3.46 shows that both chrysotile and C. parvum induced macrophages had an approximately similar rate of capping, reaching a plateau by 20

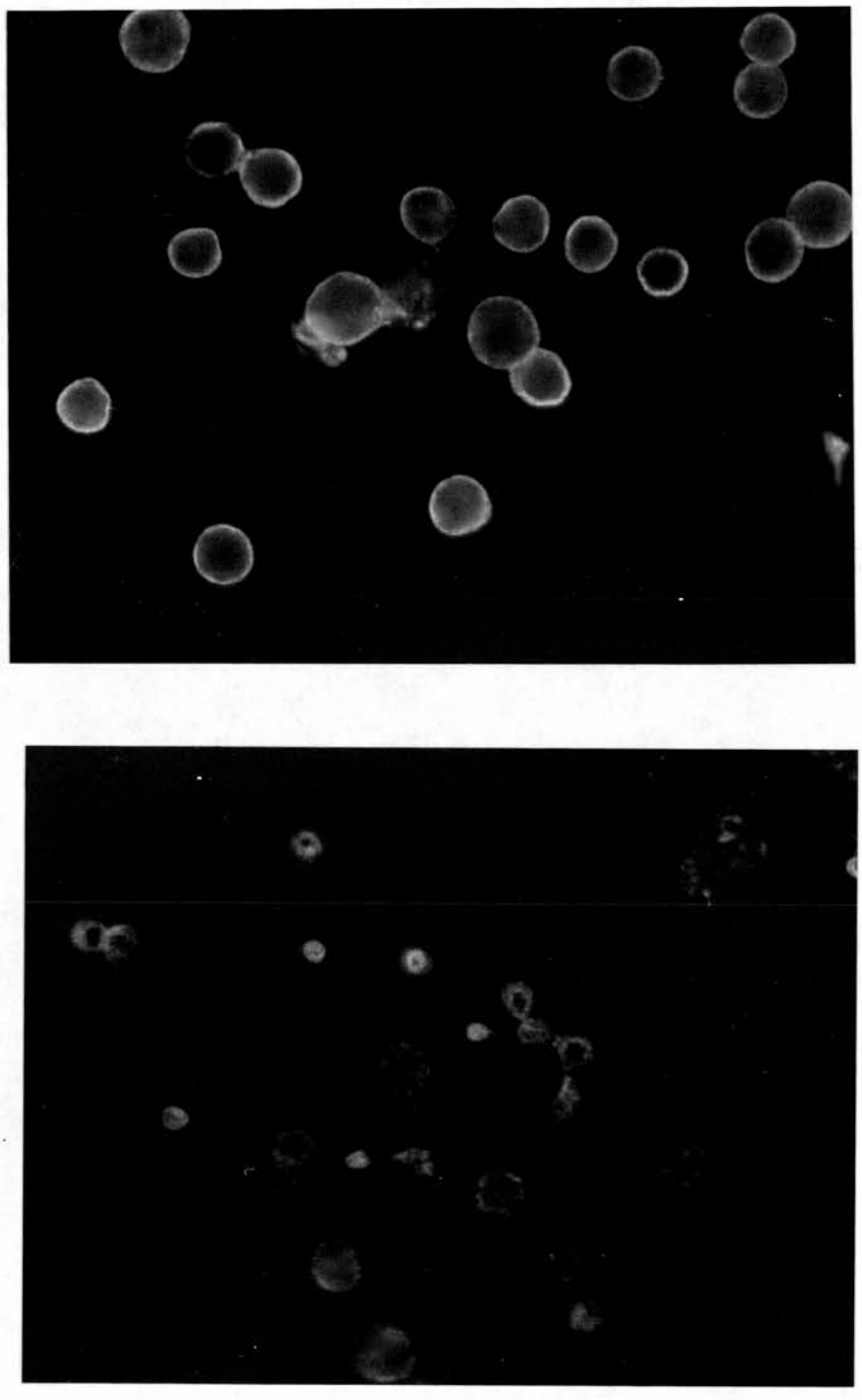


FIGURE 3.44 Upper: saline induced macrophages treated with FITC-Con A for 30 minutes at 37°C. Fluorescence is even and ring-like. Lower: chrysotile induced macrophages showing "caps" in a proportion of cells. Mag. x 600

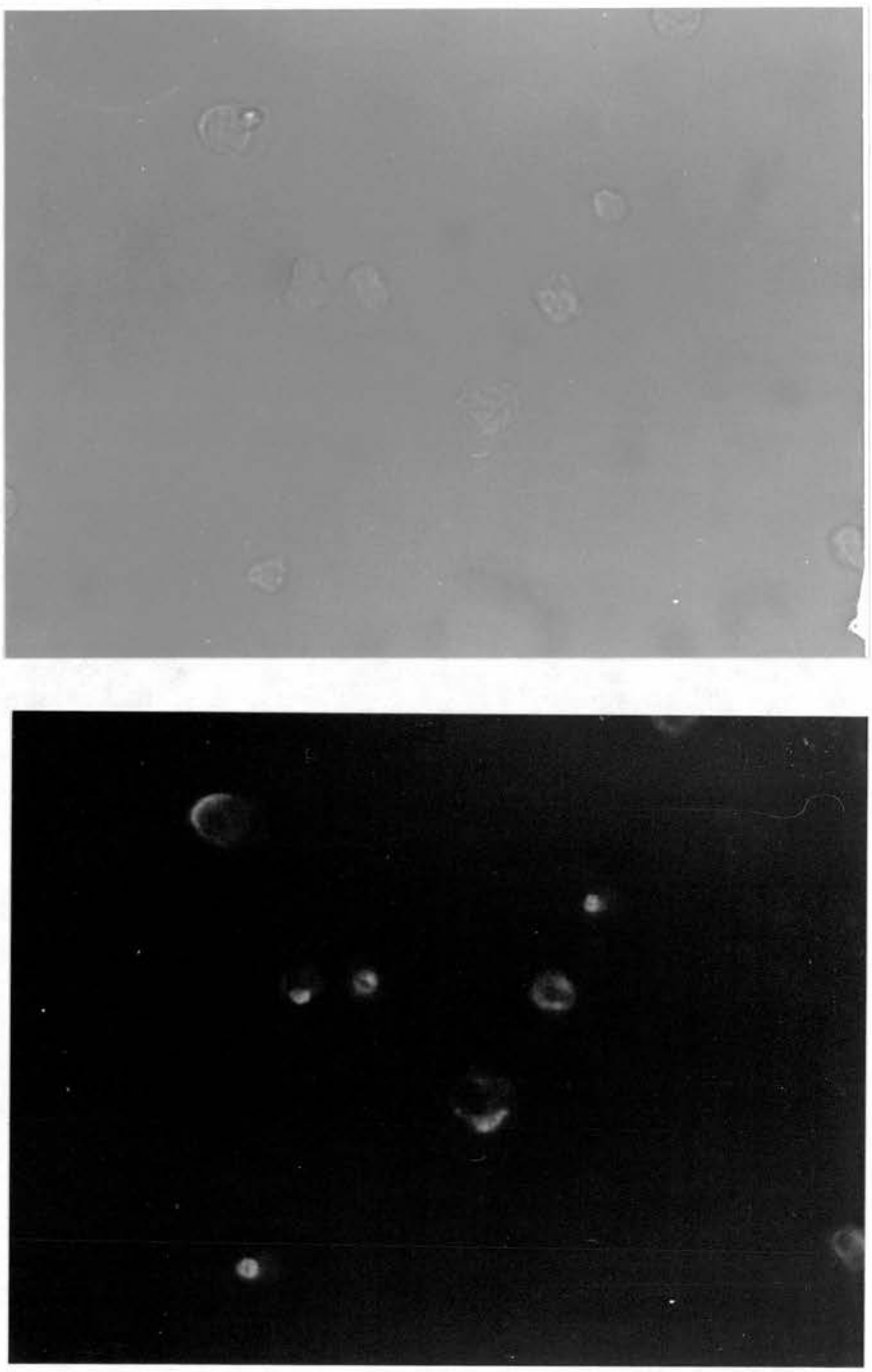


FIGURE 3.45 Bright field (upper) and fluorescence (lower) illuminated field of chrysotile induced macrophages to show capping macrophages.

Mag. x 600

TABLE 3.3 Effect of injection of different agents on proportion of capping macrophages (FITC-Con A 37°C 20 minutes).
Significant differences 3 day saline v 3 day chrysotile, 3 day crocidolite and 5 day C. parvum - $P < 0.001$;
3 day chrysotile v 3 day crocidolite - $P < 0.02$.

MACROPHAGE SOURCE	PERCENT CAPPING WITH FITC-CON A	NUMBER OF EXPERIMENTS
3 day saline	4.1 ± 2.3	8
3 day chrysotile	32.2 ± 4.2	5
3 day crocidolite	22.8 ± 2.6	3
5 day C. parvum	32.4 ± 5.0	6
3 day proteose peptone	1.2 ± 1.1	2
3 day latex	3.2 ± 0.9	1

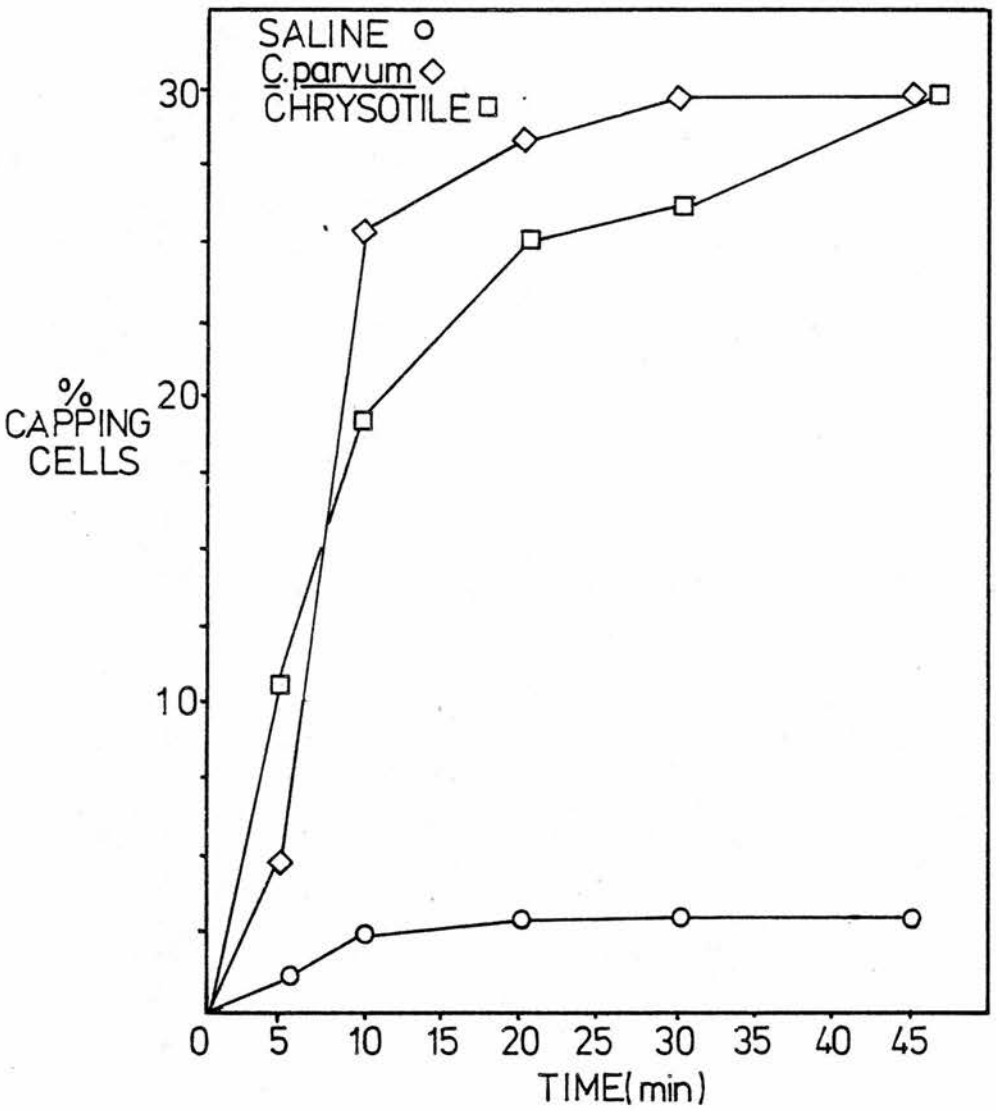


FIGURE 3.46 **Time course of capping in saline, chrysotile and C. parvum induced macrophages.**

$\bar{x}$ of duplicate coverslips: repr. exp.

minutes with little increase thereafter. It was notable that, after 30 minutes, the caps became more diffuse, as though they were internalized and no longer easily focused at the cell surface.

3.4.2.4 Temperature dependence of capping

Table 3.4 reveals the temperature dependence of capping using C. parvum activated macrophages. At room temperature the percentage of capping cells remained at control levels with a significant ($P < 0.001$) difference between C. parvum macrophages at 37°C and at room temperature with regard to percentage capping cells.

3.4.2.5 Dose response of chrysotile induced capping

Fig. 3.47 reveals that increasing doses of intraperitoneal chrysotile caused a dose dependent increase in proportion of capping macrophages which had reached a plateau by 2.5 mg, the dose used in this study.

3.4.2.6 Capping in a rapidly adhering subpopulation of activated macrophages

Table 3.5 shows the proportion of capping macrophages in 3 day saline, chrysotile and C. parvum induced macrophages adhered for 15 or 60 minutes before washing. The use of a 15 minute adhering subpopulation was aimed at trying to select out the most activated, and therefore rapidly adhering, subpopulation. The number of cells in the 15 minute adhering fraction was approximately 2/3 of the number in the 60 minute adhering fraction. It is apparent from Table 3.5 that there was no evidence of increased proportions of capping macrophages in the 15 minute adhering subpopulation compared to the 1 hour adhering population.

3.4.2.7 Pharmacological modification of capping

The microtubule disrupting drug colchicine and the microfilament disrupting drug cytochalasin B were used to pre-treat activated macrophages to ascertain the role of these cytoskeletal elements in capping. Table 3.6 shows that neither drug had any effect on the low level of capping

TABLE 3.4 Dependence of capping in C. parvum induced macrophages, on temperature.
Significant differences 5 day C. parvum 37°C v 5 day C. parvum room temperature $P < 0.001$.

MACROPHAGE SOURCE	TREATMENT	% CAPS	n
3 day saline	37°C 20 min.	4.1 ± 2.3	8
5 day C. parvum	37°C 20 min.	32.4 ± 5.0	5
5 day C. parvum	Room temp. 20 min.	1.8 ± 0.8	3

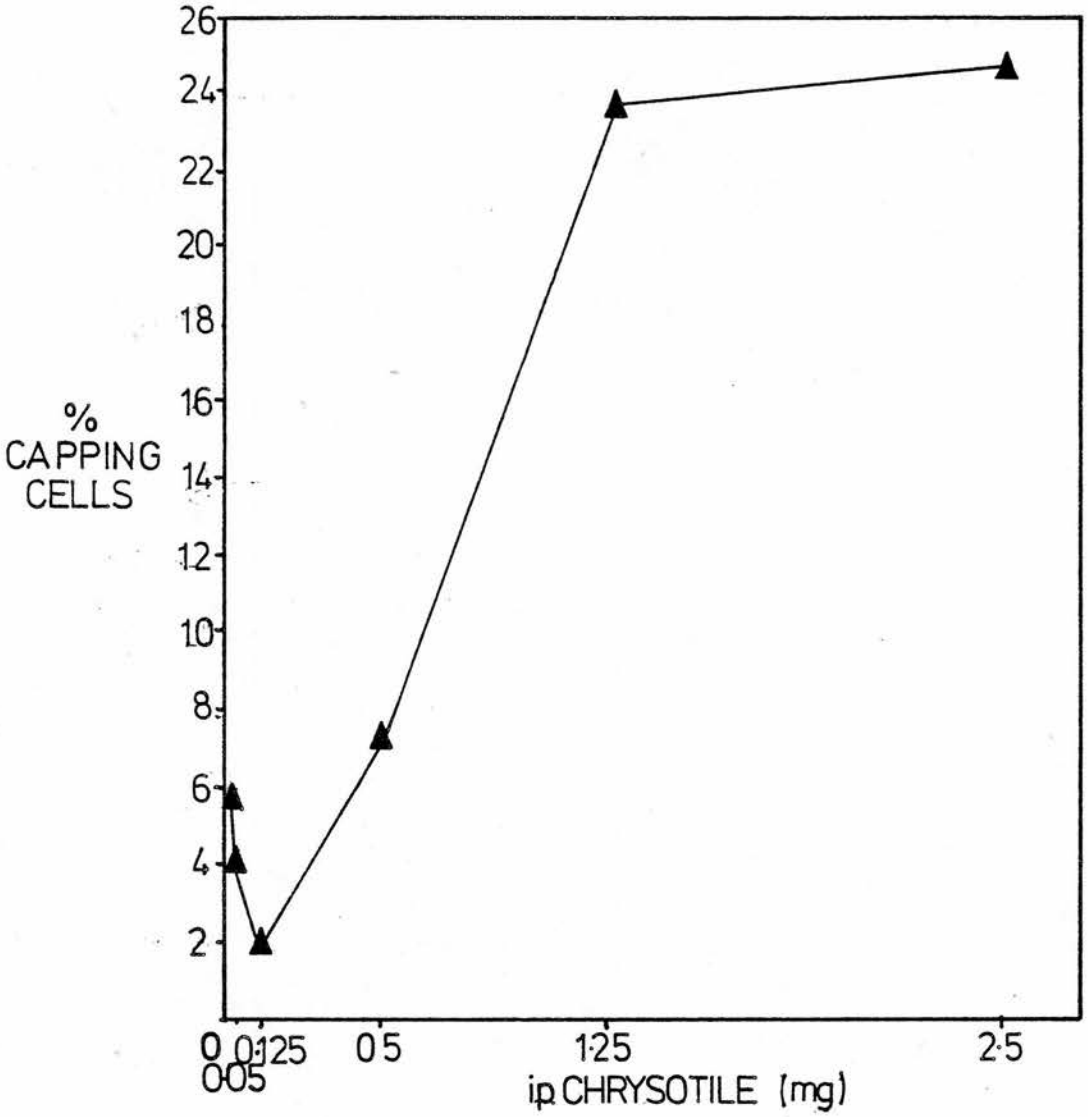


FIGURE 3.47 **Effect of increasing the intraperitoneal dose of chrysotile on the proportion of macrophages capping with Con A.** $\bar{x}$ of 2 mice at each dose

TABLE 3.5 Proportion of capping macrophages in 15 and 60 minute adhering populations of saline, chrysotile and C. parvum induced macrophages.

MACROPHAGE SOURCE	% CAPPING CELLS	
	15 MINUTE ADHERENT	60 MINUTE ADHERENT
3 day saline	1.0 ± 2.3	4.2 ± 1.9
3 day chrysotile	26.4 ± 5.2	33.2 ± 4.1
5 day C. parvum	30.0 ± 1.8	33.5 ± 6.8

$\bar{x} \pm sd$ of triplicate c/slips

TABLE 3.6 Effect of pretreatment with saline, colchicine or cytochalasin B on Con A capping in 3 day saline and chrysotile induced macrophages.

Significant difference:- 3 day chrysotile, saline v cytochalasin B treatment $P < 0.001$.

MACROPHAGE SOURCE	PRE-TREATMENT*	% CAPPING CELLS ($\bar{x} \pm sd$)	n
3 day saline	saline	4.2 ± 1.6	3
"	10^{-4}M colchicine	5.6 ± 4.8	4
"	10^{-4}M cytochalasin B	3.0 ± 5.2	4
3 day chrysotile	saline	32.7 ± 4.1	3
"	10^{-4}M colchicine	39.5 ± 10.5	3
"	10^{-4}M cytochalasin B	6.0 ± 2.9	3

*Pretreatment was 1 hour at 37°C.

present in 3 day saline induced macrophages. In the 3 day chrysotile induced macrophages there was no significant difference between saline and colchicine pre-treated macrophages with regard to percentage of capping cells. Cytochalasin B pre-treatment did, however, significantly ($P < 0.001$) reduce the percentage of capping chrysotile induced macrophages down to control levels.

Cytochalasin B was prepared by diluting first in dimethyl-sulphoxide and so the effect of dimethyl-sulphoxide alone on capping was tested. As shown in Table 3.7 dimethyl-sulphoxide alone had no effect on capping while cytochalasin B containing the same amount of dimethyl-sulphoxide reduced the proportion of capping cells to less than 25% of the saline treated control.

Lignocaine is a local anaesthetic known to act against the cytoskeleton and so the effect of this drug on capping was measured. As shown in Table 3.8 lignocaine pre-treatment also reduced capping although this result was confused by detachment of up to 50% of macrophages from the coverslip by the time they were mounted.

3.4.2.8 Re-expression of Con A receptors following occupation of receptors

Saline induced macrophages showed re-expression of bright ring fluorescence 3-4 hours after treatment with unlabelled Con A (Fig. 3.48) Chrysotile induced macrophages also showed re-appearance of ring fluorescence in the uncapped cells and bright fluorescent caps in a subpopulation of cells within the same time interval.

3.4.2.9 Capping in nude mice

Nude mice have been reported to have peritoneal macrophages in a perpetual state of activation (Zinkernagel and Blanden 1975) and so unstimulated peritoneal macrophages from nude mice were obtained and tested for Con A capping compared to saline induced macrophages from non-nude mice. As shown in Table 3.9 nude mice had a significantly

TABLE 3.7 Effect of saline, DMSO or cytochalasin B pre-treatment on the proportion of capping cells in chrysotile induced macrophages ($\bar{x}$ of duplicate coverslips).

PRE-TREATMENT[+]		
SALINE	1% DMSO	CYTOCHALASIN B 10^{-4}M in 1% DMSO
39.8	35.8	9.3

[+]Pre-treatment was 1 hour at 37°C.
$\bar{x}$ of duplicate c/slips

TABLE 3.8 Effect of lignocaine pre-treatment on percent of capping macrophages.

PRE-TREATMENT[+]	
SALINE	LIGNOCAINE[*]
28.6 ± 6.2	6.9 ± 0.8

[+]Pre-treatment was 1 hour at 37°C.
[*]10^{-2}M lignocaine.
[‡]

TABLE 3.9 Percentage of capping macrophages in macrophages from CBA and CBA nu/nu mice.

CBA/Ca[+]	CBA nu/nu[*]
4.1 ± 2.8	10.5 ± 2.2

Significant difference $P<0.01$
[+]3 day saline induced
[*]unstimulated
[‡]
[‡] $\bar{x} \pm$ sd of triplicate c/slips

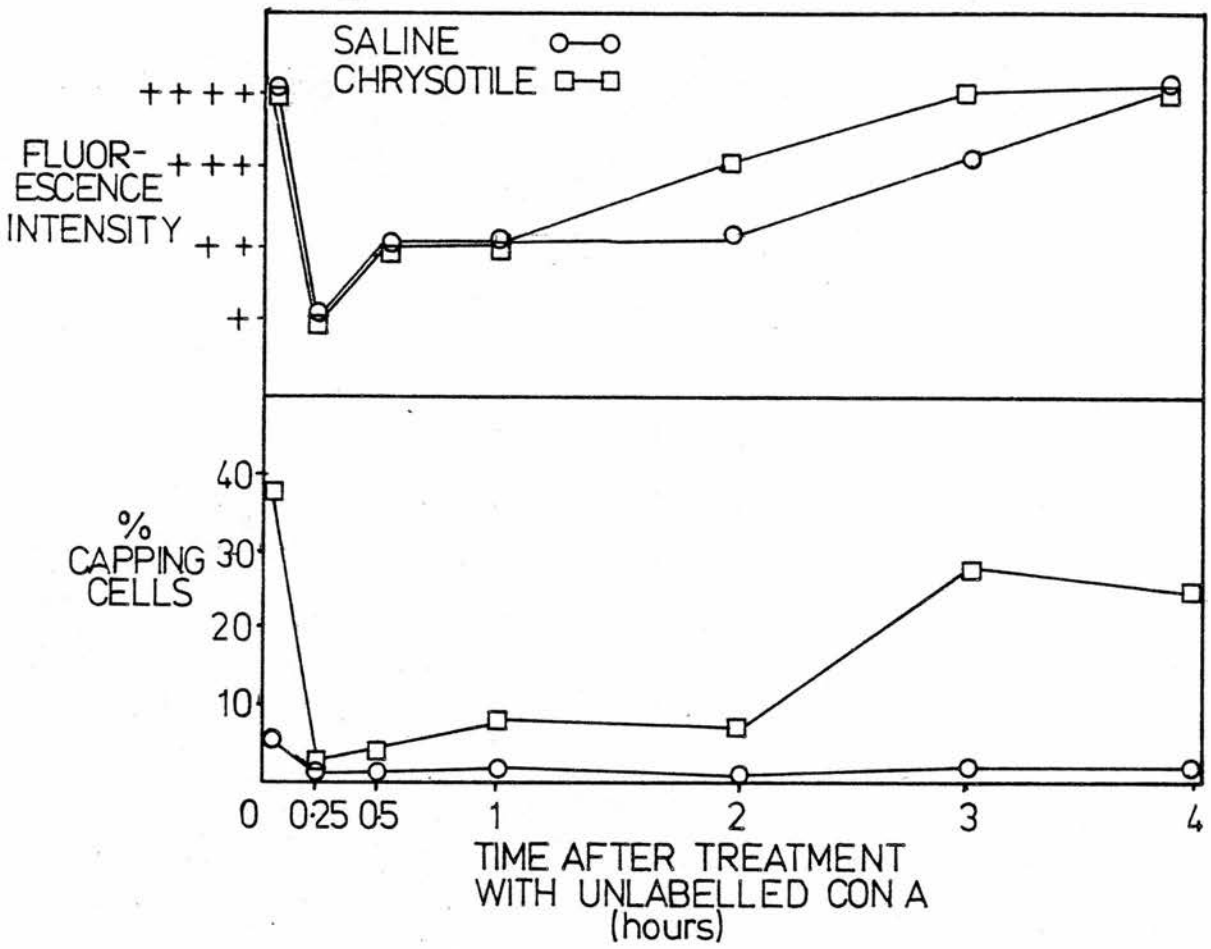


FIGURE 3.48 **Reappearance of membrane Con A fluorescence with time following treatment with unlabelled Con A in saline and chrysotile induced macrophages.**

$\bar{x}$ of duplicate c/s: repr. exp.

($P < 0.01$) greater proportion of capping macrophages than saline induced macrophages from normal mice.

3.4.2.10 Electron microscopy of capping cells

Saline and chrysotile induced macrophages were fixed for electron microscopy before and after treatment with Con A under capping conditions.

(i) Scanning electron microscopy

The appearance of 3 day saline induced macrophages at SEM has been described (Section 3.2.6.3; Figs. 3.15).

On treatment with Con A for 20 minutes at 37°C there was a striking alteration in the membrane configuration of 25-50% of the macrophages and this was apparent as a change from the low ruffles seen in Fig. 3.15 to large frond-like processes shown in Fig. 3.49; as is apparent from Fig. 3.49 (lower) some macrophages remained largely unaffected by Con A treatments.

Chrysotile induced macrophages did not show such a marked increase in membrane ruffling as did the saline induced macrophages on treatment with Con A; a proportion of them did, however, have large conspicuous blebs at one pole (Fig. 3.50) which could be homologous with the caps seen at fluorescence microscopy. The difference in tone of the "bleb" compared to the rest of the cell in Fig. 3.50 suggests that this region of membrane has a different secondary electron emission to the rest of the cell membrane and so tends to confirm that the physicochemical nature of the bleb membrane is different. This presumably reflects the concentration of Con A and its glycoprotein receptor in the bleb. It was also notable that more chrysotile induced macrophages had spread following Con A treatment.

(ii) Transmission electron microscopy of capped chrysotile macrophages

Three day chrysotile induced PEC were brought into siliconised tubes and treated with Con A under capping conditions then processed as a

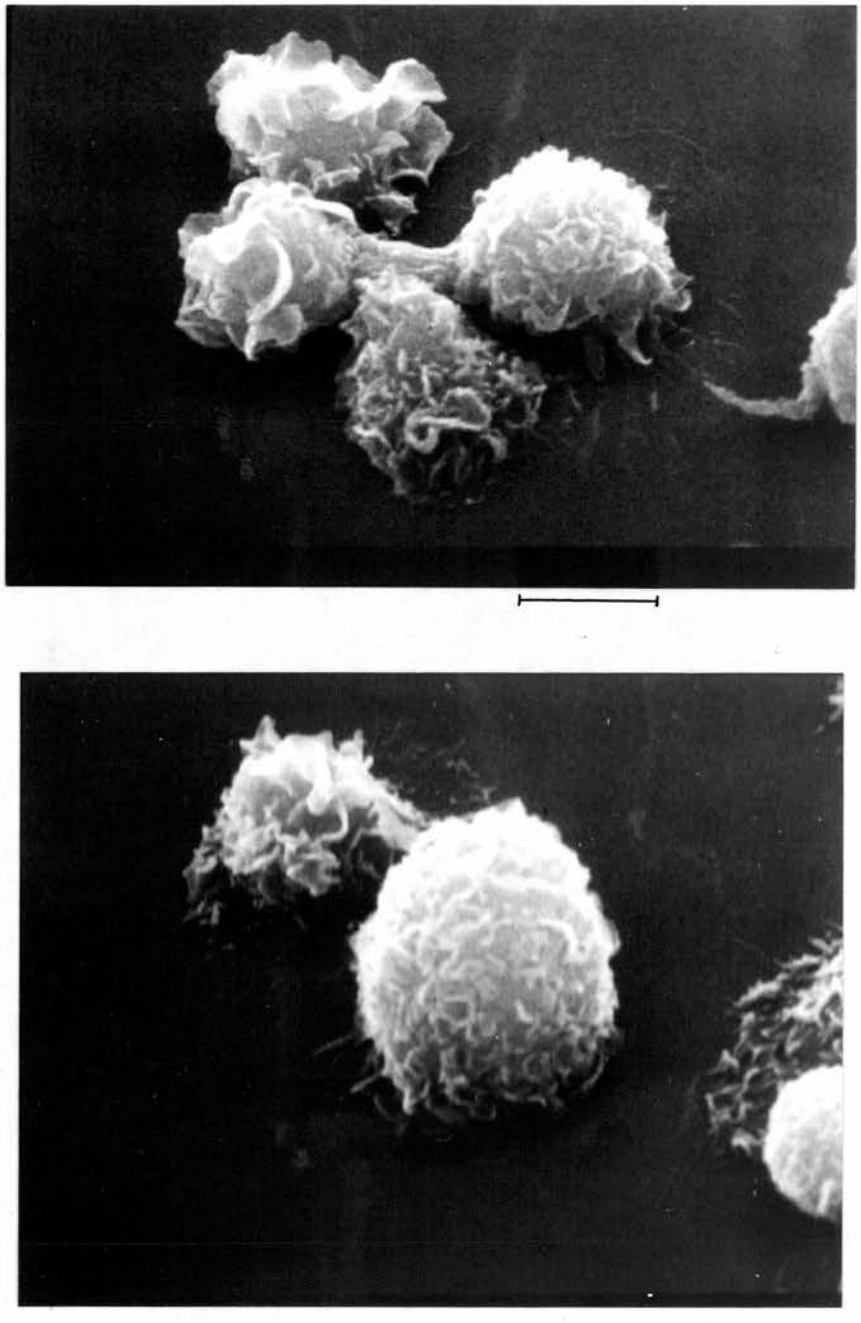


FIGURE 3.49 Scanning electron micrographs of saline induced macrophages treated with Con A. Marker line 4 μm.

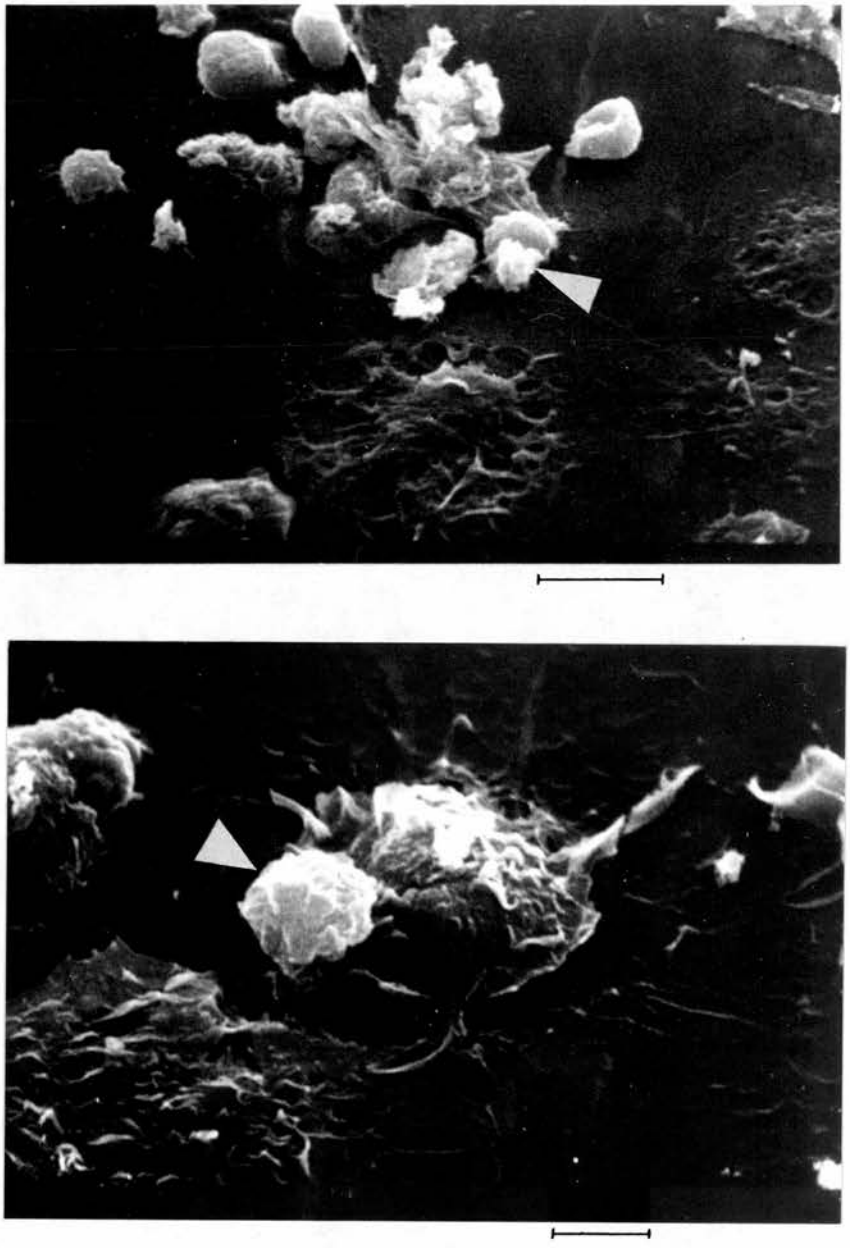


FIGURE 3.50 Scanning electron micrographs of chrysotile induced macrophages incubated with Con A. Large "blebs" (arrows) showing secondary electron emission different from the rest of the cell may represent caps.
Marker line:- upper 10 μm; lower 2 μm.

pellet for TEM. Despite a comprehensive search no structural correlate could be seen equivalent to the caps seen by light and scanning electron microscopy. It is clear from Figs. 3.15 and 3.16 that 3 day chrysotile induced macrophages have exceedingly complex surface infoldings. On treatment with Con A this infolding and vacuolation was, on a qualitative basis, seen to be slightly increased (Fig. 3.51) and the number of cells which could be scored as having this pattern of surface membrane infolding (in 200 cells assessed at 2,000 x magnification) rose from 33.6% in non-Con A treated to 59.7% in Con A treated. No clear region corresponding to a "cap" could be seen and this could be due to at least two possibilities:-

(i) capping was always carried out, in the light and SEM experiments discussed above, on adherent cells while for TEM the capping was carried out on suspension where the occurrence of caps was never investigated;

(ii) if 30% of macrophages have caps, whole PEC comprising 65% macrophages will have only 20% of all cells with caps; the likelihood of sectioning through a cap in a randomly oriented cell could be conservatively guessed at 50:1; thus in 100 PEC sectioned it could be predicted that only 1-2 macrophage caps would be visible on section.

3.4.3 Use of $[^{125}I]$ Con A to assess the number of Con A binding sites in the macrophage membrane

3.4.3.1 Effect of phagocytosis of latex spheres on $[^{125}I]$ Con A binding

The binding of $[^{125}I]$ Con A to macrophages which had phagocytosed latex spheres was significantly ($P < 0.001$) less than binding to control macrophages as shown in Table 3.10. The latex treated macrophages had 42.2% of the binding to untreated macrophages.

3.4.3.2 Attempts to measure the effect of phagocytosis of antibody coated SRBC on $[^{125}I]$ Con A binding

This method necessitated the lysis of the surface adherent

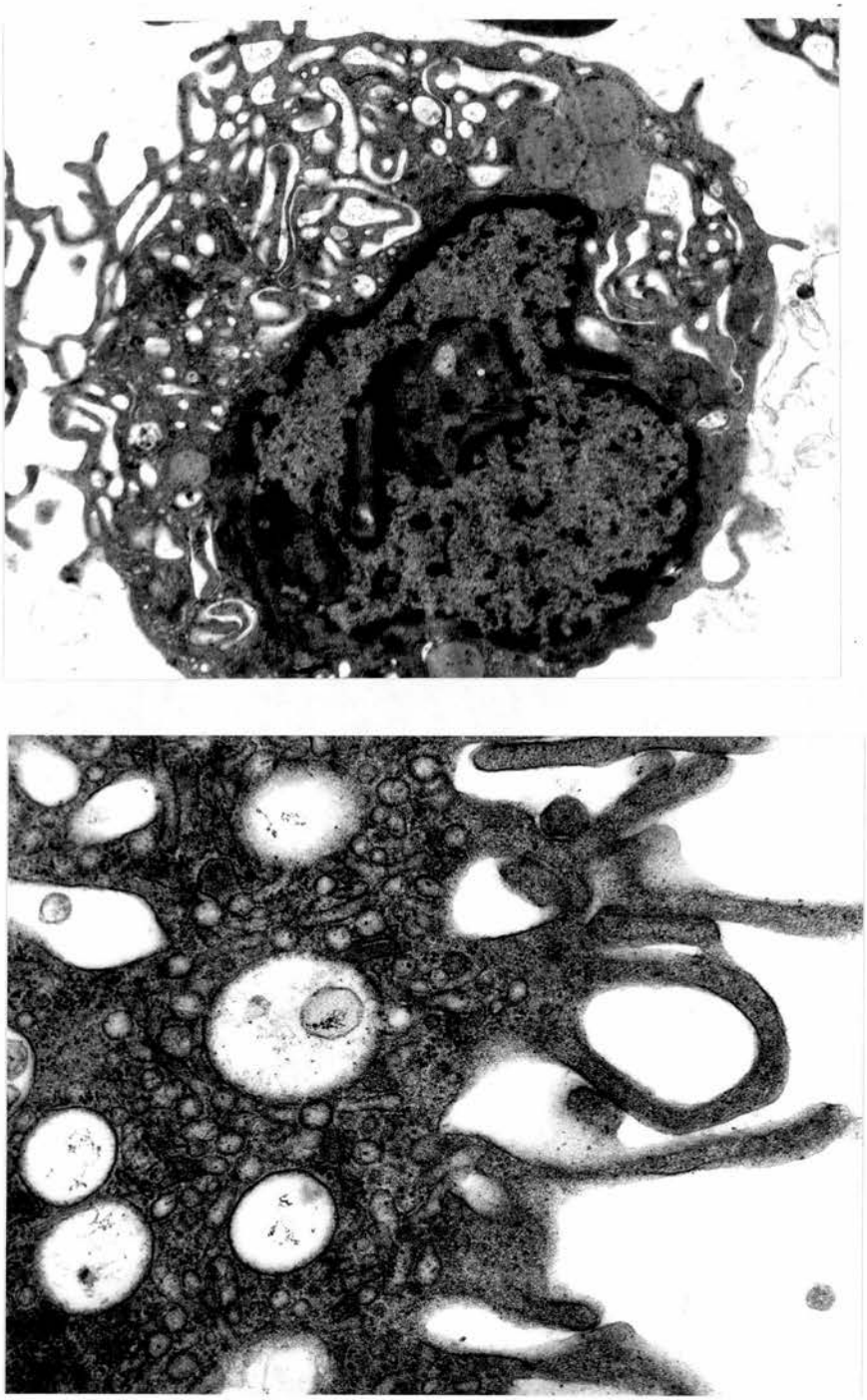


FIGURE 3.51 Transmission electron micrographs of chrysotile induced macrophages incubated with Con A under capping conditions.
Upper - low power view to show general increase in membrane activity compared with Fig. 3.11.
Lower - high power view of membrane and associated vesicle traffic.
Mag. - upper x 7,600; lower x 18,500.

TABLE 3.10 Binding of $[^{125}I]$ Con A to control macrophages treated with Dul A or latex spheres.
Significant difference $P<0.001$.

TREATMENT	BINDING OF $[^{125}I]$ CON A (cpm $\bar{x}\pm$ sd)
Saline	$28,046\pm3,945$
Latex	$16,227\pm2,515$

*

TABLE 3.11 Binding of $[^{125}I]$ Con A to control macrophages, control macrophages treated with lytic buffer and control macrophages treated with SRBC then lytic buffer.
Significant differences - control v control + lytic buffer, $P<0.001$; lytic buffer v SRBC + lytic buffer, $P<0.05$.

TREATMENT	BINDING OF $[^{125}I]$ CON A (cpm $\bar{x}\pm$ sd)
Control	$21,779\pm1,480$
Lytic buffer	$11,661\pm3,067$
SRBC then lytic buffer	$7,890\pm1,736$

Blank $4,850\pm274$

*

* $\bar{x}\pm$sd of 4-10 c/s: repr. exp^{t}

unphagocytosed SRBC's so that they would not interfere by binding $[^{125}I]$ Con A at the macrophage surface. However, as shown in Table 3.11, treatment of the control macrophages with the lytic buffer alone resulted in a significant ($P < 0.001$) reduction in $[^{125}I]$ Con A binding compared to control macrophages treated with Dul A. Thus although there was a reduction in binding by 30% ($P < 0.05$) this was over and above a 47% reduction in binding due to lytic buffer alone.

3.4.3.3 Binding of $[^{125}I]$ Con A following phagocytosis of asbestos

The binding of $[^{125}I]$ Con A to macrophages following phagocytosis of both crocidolite and chrysotile asbestos was always greater than the binding to controls. Table 3.12 shows the results of an experiment where crocidolite phagocytosis resulted in a 20% increase in binding of $[^{125}I]$ Con A, while Table 3.13 shows an increase in binding of 8.5% following phagocytosis of chrysotile. In 5 separate experiments using asbestos, reduction in binding was never found following phagocytosis and increases ranged from 8.5-23.1% over controls. Microscopic examination of cells confirmed that phagocytosis had occurred in these experiments and it was assumed that cell surface adherent fibres and incompletely phagocytosed fibres were responsible for the increased binding.

3.4.3.4 $[^{125}I]$ Con A binding to control macrophages and macrophages elicited with asbestos or C. parvum

The binding of $[^{125}I]$ Con A to equal numbers of control, asbestos or C. parvum activated macrophages are shown in Table 3.14 and while saline and chrysotile elicited macrophages both bound similar amounts of $[^{125}I]$ Con A the C. parvum elicited macrophages bound significantly more (34.0%; $P < 0.002$).

TABLE 3.12 Effect of crocidolite treatment on binding of $[^{125}I]$ Con A to control macrophages.

TREATMENT	BINDING OF $[^{125}I]$ CON A (cpm $\bar{x}\pm$ sd)
Saline	$16,132\pm2,933$
Crocidolite	$20,123\pm4,220$

Blank $4,402\pm1,346$

*

TABLE 3.13 Effect of chrysotile treatment on binding of $[^{125}I]$ Con A to control macrophages.

TREATMENT	BINDING OF $[^{125}I]$ CON A (cpm $\bar{x}\pm$ sd)
Saline	$25,472\pm2,031$
Chrysotile	$27,832\pm3,474$

Blank $6,842\pm920$

* $\bar{x}\pm$s.d. of 4-10 c/s: repr. expts.

TABLE 3.14 Binding of $[^{125}I]$ Con A to saline, chrysotile and C. parvum induced macrophages.

Significant difference - saline v C. parvum $P<0.002$

MACROPHAGE SOURCE	BINDING OF $[^{125}I]$ CON A (cpm $\bar{x}\pm$ sd)
Saline	$38,960\pm2,616$
Chrysotile	$36,361\pm2,084$
C. parvum	$52,207\pm4,855$

Blank $10,951\pm3,670$

$\bar{x}\pm$s.d. of 4-10 c/s: data from 3 separate expts.

3.4.3.5 Trypsin accessibility of [^{125}I] Con A bound to the surface of control and elicited macrophages

In order to gain information on the disposition of bound Con A in the surfaces of control or activated macrophages the protease releasable counts were determined following binding using in the first instance trypsin and pronase. As shown in Table 3.15 trypsin and pronase released the same proportion of counts from both control and chrysotile activated macrophages; trypsin was therefore used in subsequent experiments. As shown in Table 3.16 the percentage of bound [^{125}I] Con A which could be released by trypsin treatment was the same for control, chrysotile and C. parvum activated macrophages.

3.5 Effect of asbestos induced macrophage supernatants on lymphocyte mitogenesis

3.5.1 Attempts to detect Interleukin 1 activity in supernatants from macrophages activated by LPS treatment in vitro

Bacterial lipopolysaccharide (LPS) has been used extensively to activate macrophages in vitro to release Interleukin 1 (IL1) activity into the supernatant (Murphy et al 1980; Tenu et al 1980). Since earlier work in this study had revealed that asbestos could activate macrophages in vivo, it was suspected that asbestos activated macrophages might also release increased amounts of IL1 activity. To develop the IL1 assay, LPS treatment of macrophages in vitro was carried out in the hope of obtaining a standard IL1-rich supernatant. Control macrophages were treated with LPS for 24 and 72 hours before collecting supernatants which were tested in a thymocyte mitogenesis assay. As shown in Tables 3.17 and 3.18 supernatants had no enhancing effect on sub-optimal Con A mitogenesis and therefore no evidence of IL1 activity; this held true for both 72 and 96 hour assays.

TABLE 3.15 Percentage of $[^{125}I]$ Con A released from saline and chrysotile induced macrophages by trypsin and pronase.

MACROPHAGE SOURCE	% COUNTS RELEASED	
	TRYPSIN	PRONASE
3 day saline	27.2 ± 2.3	30.7 ± 1.8
3 day chrysotile	30.2 ± 0.6	31.5 ± 0.9

*

TABLE 3.16 Percent of $[^{125}I]$ Con A released by trypsin from $[^{125}I]$ Con A treated macrophages.

MACROPHAGE SOURCE	PERCENTAGE OF TRYPSIN RELEASED COUNTS
3 day saline	31.9 ± 2.9
3 day chrysotile	33.2 ± 1.3
5 day C. parvum	30.7 ± 2.4

*

* $\bar{x} \pm$ s.d. of 3 separate exp^{s}.

TABLE 3.17 Effect on thymocyte mitogenesis of supernatants from macrophages treated with LPS in vitro for 24 hours. Label added on Day 2 or Day 3 of assay.

		DOSE OF CON A				
		0	0.5	1	2	4
LABEL ON DAY 2	Con A only	810±99*	87±19	2671±75	17836±2102	29362±8017
	Con A + Supernatant	130±46	306±159	1513±146	10442±946	23584±1479
LABEL ON DAY 3	Con A only	192±100	374±131	4078±851	14822±1407	19281±1001
	Con A + Supernatant	293±194	244±127	2494±652	10596±1292	18580±1033

*cpm $\bar{x}\pm$ sd. †

TABLE 3.18 Effect on thymocyte mitogenesis of supernatants from macrophages treated with LPS in vitro for 72 hours. Label added on Day 2 and Day 3 of assay.

		DOSE OF CON A				
		0	0.5	1	2	4
LABEL ON DAY 2	Con A only	62±13*	94±25	803±204	5296±601	22244±4559
	Con A + Supernatant	220±135	296±95	902±358	5561±841	15970±873
LABEL ON DAY 3	Con A only	102±12	122±34	2312±343	7414±1237	22758±9844
	Con A + Supernatant	197±219	291±110	1824±812	7712±984	14586±1787

*cpm $\bar{x}\pm$ sd. † triplicate wells : repr. expt

3.5.2 Attempts to detect IL1 activity in supernatants from macrophages activated by in vivo treatment

Both proteose peptone and Con A have been used to induce activated macrophages in the peritoneal cavity and so macrophages obtained 3 days after intraperitoneal injection of both agents were plated out, the supernatants harvested, dialysed and used in the sub-optimal Con A mitogenesis assay. It is clear from Table 3.19 that the dialysed supernatants had no IL1 activity whether obtained 24 or 48 hours after plating Con A or proteose peptone induced macrophages in serum-free or serum containing medium.

In view of these failures to detect IL1 activity in supernatants from macrophages activated in conventional ways, and in view of the evidence from some of these experiments (e.g. Table 3.18) that suppression of Con A mitogenesis could be a property of some of these supernatants, the literature was reviewed and abundant evidence for suppressor activity release by activated macrophages was found (e.g. Nelson 1976; Schechter et al 1980). An asbestos induced macrophage supernatant was prepared and found to exhibit suppressor activity and so standard supernatants were prepared from 3 day saline, latex, crocidolite and chrysotile induced macrophages and their properties investigated.

3.5.3 Effect of Con A dose on detection of suppressor activity

Fig. 3.52 shows the results of an experiment where 3 day saline and chrysotile macrophage supernatants were tested in the Con A thymocyte mitogenesis assay. A general effect is apparent, which was clear from early experiments, that the suppressor effect of the activated macrophage supernatant was maximal at 1 or 2 μg/ml Con A. In most experiments 1 and 2 μg/ml Con A were the doses used to detect suppressor activity.

TABLE 3.19 Effect on Con A mitogenesis of supernatants collected after 24 or 48 hours incubation of macrophages induced with proteose peptone (pp) or Con A in the presence or absence of Foetal Calf Serum (FCS). All supernatants dialysed overnight against RPMI at 4°C.

	CONTROL	24 h pp No FCS	24h pp FCS	24h Con A No FCS	24h Con A FCS	48h Con A No FCS	48h Con A FCS
No Con A	690±129*	556±51	578±175	647±122	494±47	306±71	675±203
Con A	880±162	568±48	598±48	650±128	757±235	406±65	406±61

*cpm $\bar{x}$ ± sd (triplicate wells: repr. expt).

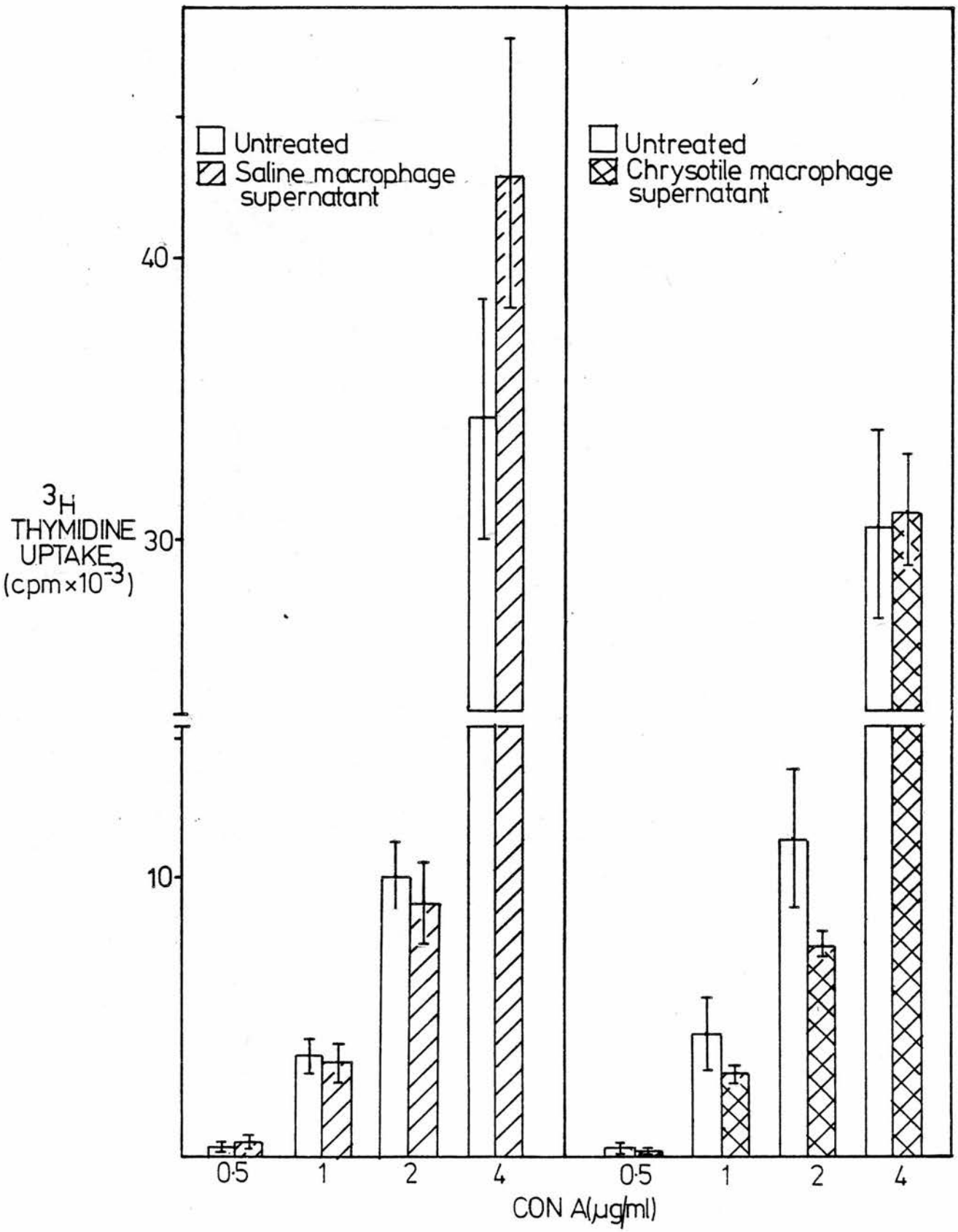


FIGURE 3.52 **Effect of saline and chrysotile induced macrophage supernatant on Con A mitogenesis of thymocytes.**
Symbols denote $\bar{x} \pm$ one standard deviation (triplicate wells: repr. exp.)

3.5.4 Suppressor effects of asbestos induced macrophage supernatants

Table 3.20 summarises the experiments using supernatants from macrophages induced with asbestos, saline and latex in the Con A mitogenesis assay at 1 and 2 μg/ml Con A. The results are expressed both as mean ± standard deviation of cpm of thymidine incorporated for all experiments, and as mean ± standard deviation of mitogenic index obtained for each experiment. The mitogenic index was calculated in the conventional way relating cpm due to any dose of Con A and cpm produced by the same Con A dose in the presence of supernatant (see Table 3.20). Comparing the data expressed as cpm the mean cpm with saline induced macrophage supernatant were seen to be very similar to cpm with Con A only, while with the asbestos induced macrophage supernatants the cpm were considerably less; latex supernatant at 1 μg/ml Con A also had a mean which was less than that of saline macrophage supernatant at that dose. There was, however, no significant difference in thymidine incorporation, expressed as cpm, with asbestos supernatants (or latex) compared to either Con A alone or to mitogenesis in the presence of saline macrophage supernatant. Statistical significance was confounded by variation between experiments in mitogenic response of different batches of thymocytes. The mitogenic index can be used to take into account this well documented variation in mitogenic response between experiments (Herberman 1978). When these results were expressed as mitogenic indices for each experiment there was no significant difference between latex macrophage supernatants and saline macrophage supernatants; however, crocidolite macrophage supernatants at 1 and 2 μg/ml, and chrysotile macrophage supernatants at 1 μg/ml, had significantly ($P < 0.05$ and $P < 0.02$, see Fig. 3.61) lower mitogenic indices than saline macrophage supernatants confirming significant suppressor activity in these supernatants.

TABLE 3.20 Effect of macrophage supernatants on thymocyte mitogenesis. No significant differences in tritiated thymidine uptake when expressed as cpm; when expressed as mitogenic index, derived as:-

$$\text{Mitogenic Index} = \frac{\text{cpm in presence of sup}^{t} + \text{mitogen}}{\text{cpm in presence of mitogen only}}$$

significant suppressive effects produced by asbestos supernatants compared to saline macrophage supernatants. Significant differences compared to saline - Chrysotile 1 μg/ml Con A $P<0.02$, 2 μg/ml $P<0.05$. Crocidolite 1 μg/ml $P<0.05$.

TREATMENT	CON A μg/ml	cpm ($\bar{x}\pm$ sd)	MITOGENIC INDEX $\bar{x}\pm$ sd
-	1	5,078±2336	-
	2	18,634±8705	-
SALINE + MACROPHAGE SUPERNATANT	1	5,155±1808	1.10±0.30
	2	19,054±7638	1.07±0.29
LATEX * MACROPHAGE SUPERNATANT	1	3,620±1966	0.76±0.23
	2	18,609±5759	0.89±0.15
CHRYSOTILE+ MACROPHAGE SUPERNATANT	1	3,694±835	0.59±0.14
	2	12,804±4112	0.65±0.16
CROCIDOLITE* MACROPHAGE SUPERNATANT	1	3,561±1128	0.75±0.14
	2	14,391±5860	0.81±0.24

+ Data for 6 separate experiments

* Data for 3 separate experiments

3.5.5 Dose response of suppression by asbestos induced macrophage supernatants

Fig. 3.53 shows the effect on thymocyte mitogenesis of a 3 day chrysotile macrophage supernatant present as 5, 25 and 50% of the total incubation volume in each well. There is a clear dose response in the mean cpm of tritiated thymidine associated with the thymocytes and even at 5% there is evidence of suppression. Significant differences between supernatant treated and control (cRPMI treated) wells were present with 50% supernatant ($P < 0.02$ and $P < 0.05$).

3.5.6 Effect of macrophage supernatants from mice injected with asbestos long term

The following experiments were carried out in order to determine whether mitogenesis suppressing factors were released only by macrophages in the acute (3 day) phase following asbestos exposure or whether long term dusted animals would also yield macrophages capable of releasing such factors. Mice were available which had been kept 280 days after injection of crocidolite in order to determine fibrogenic response and so 2 of these mice were killed, peritoneal macrophages pooled, and 24 hour supernatants prepared from the macrophages. These 280 day crocidolite macrophage supernatants were tested alongside 3 day crocidolite macrophage supernatants in the mitogenesis assay. Control mice injected alongside the long term crocidolite mice (280 day saline macrophage supernatant) served as a control for ageing effects. Fig. 3.54 shows the results of an experiment where, as expected, 3 day chrysotile macrophage supernatant was significantly suppressive to Con A mitogenesis at 1 μg/ml ($P < 0.001$) and 2 μg/ml Con A ($P < 0.01$); 280 day crocidolite supernatant, however, was not suppressive and in fact showed a significant degree ($P < 0.002$) of stimulatory activity at 1 μg/ml Con A but was not significantly different from the control at 2 μg/ml Con A. These effects of 280 day

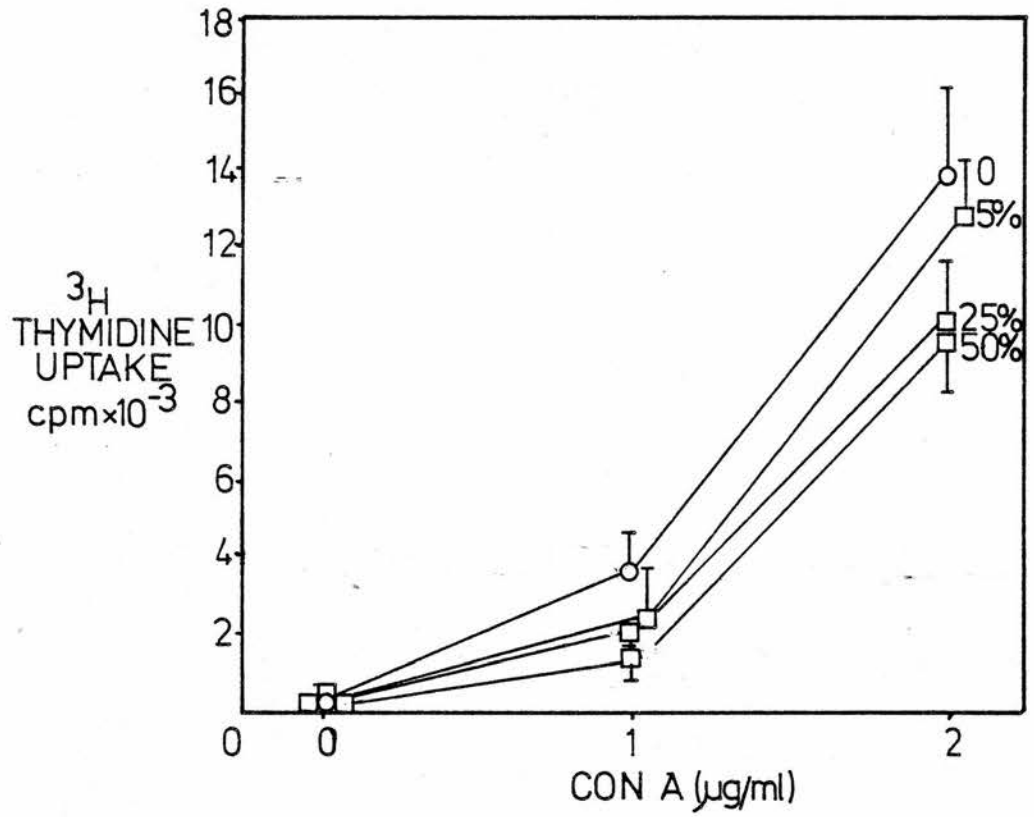


FIGURE 3.53 **Effect of chrysotile induced macrophage supernatant present in the incubation as 5, 25 or 50%, on Con A mitogenesis of thymocytes. Significant reduction with 50% supernatant at 1 µg/ml (P<0.02) and 2 µg/ml (P<0.05) Con A.** ($\bar{x}$±sd: repr.^{ve} exp^{t})

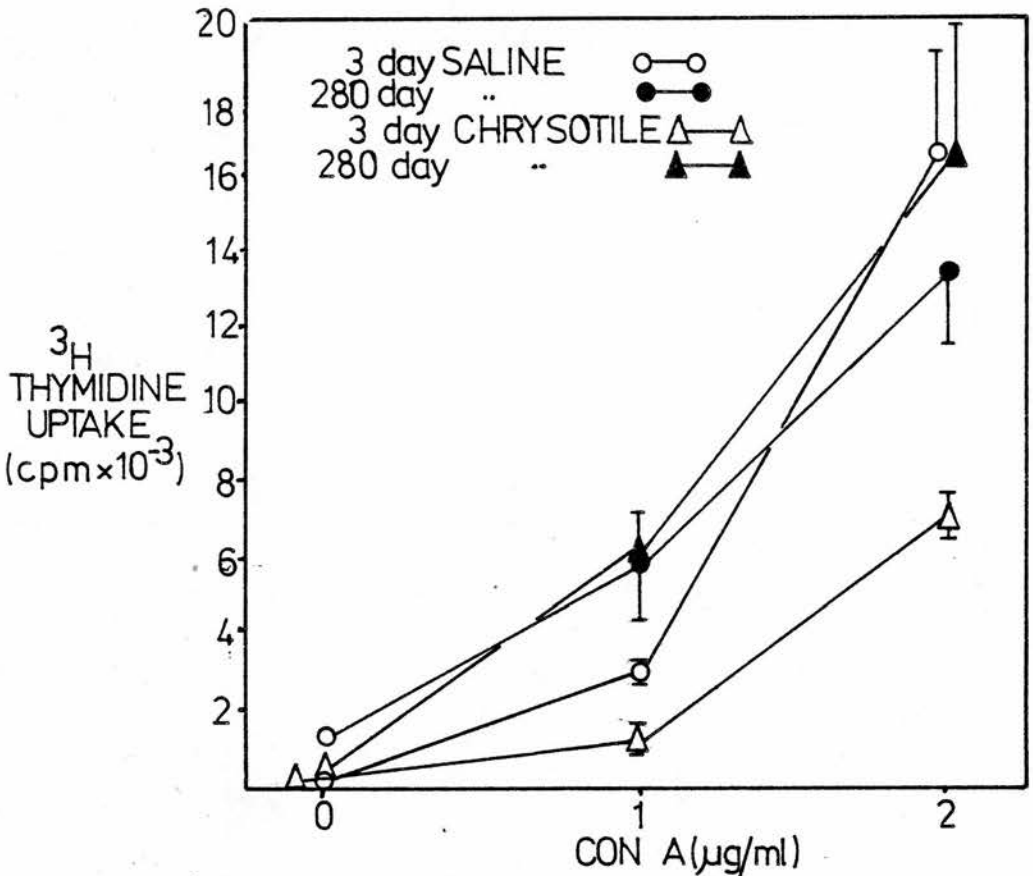


FIGURE 3.54 Effect on Con A mitogenesis of 3 day and 280 day saline and chrysotile induced macrophage supernatants. Bars denote $\bar{x} \pm$ one standard deviation. Significant reductions compared to 3 day saline produced by 3 day chrysotile at 1 μg/ml ($P<0.001$) and 2 μg/ml ($P<0.01$) Con A; significant stimulation of mitogenesis produced by 280 day chrysotile compared to 3 day chrysotile at 1 μg/ml Con A ($P<0.002$); significant stimulation of mitogenesis produced by 280 day saline compared to 3 day saline at 1 μg/ml Con A ($P<0.05$). (representative exp$^{t.}$)

crocidolite macrophage supernatants were independent of the effects of asbestos since the same effects were produced by 280 day saline macrophage supernatants, i.e. significant stimulation at 1 μg/ml Con A ($P < 0.05$) and no significant difference from 3 day saline at 2 μg/ml.

These results mean that suppressor activity is present in supernatants of acute (3 day) induced macrophages but is absent from the macrophage supernatants of long term (280 days) dusted mice.

3.5.7 Effect on mitogenesis of supernatant prepared by treating control macrophages with chrysotile asbestos in vitro

In order to test whether the suppressor factor present in 3 day asbestos induced macrophage supernatant was a non-specific factor released as a result of cell damage, control macrophages were treated with asbestos *in vitro* at a dose which caused >70% killing after 3 days. The supernatant so obtained, cleared of cell debris and asbestos by centrifugation, was tested in a mitogenesis assay along with 3 day chrysotile induced macrophage supernatants. As shown in Fig. 3.55 the 3 day chrysotile supernatant was significantly suppressor to mitogenesis at 1 μg/ml ($P < 0.02$) and 2 μg/ml ($P < 0.001$) Con A; the supernatant prepared from control macrophages treated with chrysotile *in vitro* was stimulatory to mitogenesis at 1 μg/ml Con A as judged by a significant ($P < 0.02$) increase in thymidine incorporation while there was no significant difference between this supernatant and the control at 2 μg/ml Con A.

It is evident that the suppressor activity released by 3 day asbestos induced macrophages is not due to something being released as a result of asbestos induced cell damage or death since treating macrophages with asbestos *in vitro* with lethal doses of asbestos failed to cause the appearance of suppressor factors in the supernatants.

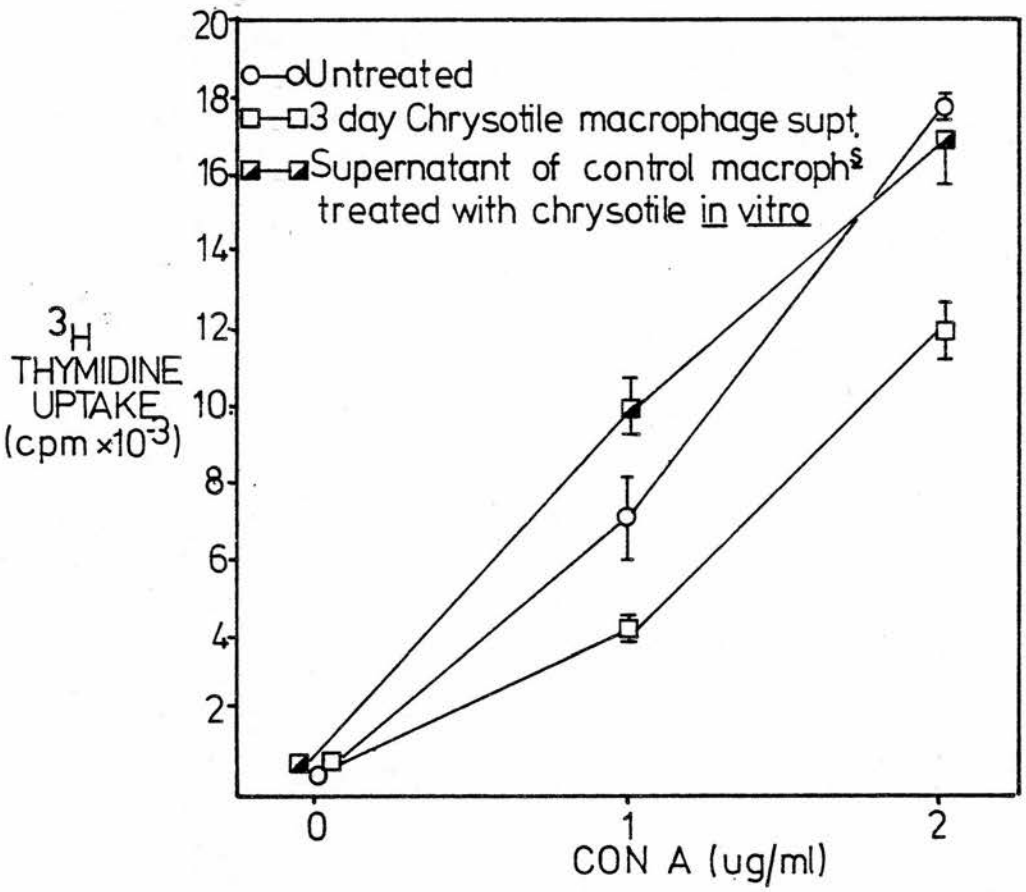


FIGURE 3.55 Effect of supernatant prepared by the in vitro treatment of control macrophages with chrysotile on thymocyte mitogenesis by Con A. 3 day chrysotile (in vivo) macrophage supernatant included as positively suppressor supernatant. Symbols denote $\bar{x} \pm$ one standard deviation. Significant inhibition of mitogenesis produced by the in vivo chrysotile supernatant at 1 μg/ml ($P<0.02$) and 2 μg/ml ($P<0.001$) Con A. Significant enhancement of mitogenesis produced by in vitro chrysotile/macrophage supernatant at 1 μg/ml Con A ($P<0.02$). (representative expt.)

3.5.8 Effect of dialysis on mitogenesis suppression by asbestos induced macrophage supernatants

Fig. 3.56 shows the effects, on 3 day chrysotile macrophage supernatant suppression, of dialysing the supernatant against medium for 24 hours. The 3 day chrysotile supernatant produced significantly less thymidine incorporation than the 3 day saline supernatant at both 1 μg/ml Con A ($P < 0.05$) and 2 μg/ml Con A ($P < 0.001$). With dialysis, however, this significance was abolished at 1 μg/ml Con A and at 2 μg/ml Con A the suppression was more than halved and, while there was still a significant difference between the dialysed chrysotile macrophage supernatant and the control, the level of significance was reduced to $P < 0.05$.

3.5.9 Effect of supernatants on a fibroblast cell line

To determine whether the suppressor activity in the 3 day asbestos macrophage supernatants had any specificity to lymphoid cells, their effect on proliferation of a fibroblast cell line was determined. As shown in Table 3.21 there was no significant difference in thymidine incorporation between untreated and 3 day saline macrophage supernatant treated fibroblasts. With the asbestos macrophage supernatants there was no significant difference between chrysotile supernatant and saline macrophage supernatant while crocidolite supernatants caused a significant ($P < 0.01$) increase in thymidine uptake.

3.5.10 Use of nylon wool purified spleen cells as targets

In order to determine whether asbestos macrophage suppressor supernatants were active against mature lymphocytes as well as thymocytes they were tested against nylon wool purified, T cell enriched, splenocytes. As shown in Table 3.22 which can be compared with the summary Table (Table 3.21) the 3 day chrysotile macrophage supernatants were rather less suppressor to splenocytes than to thymocytes but the mitogenic indices with chrysotile macrophage supernatants were still significantly less

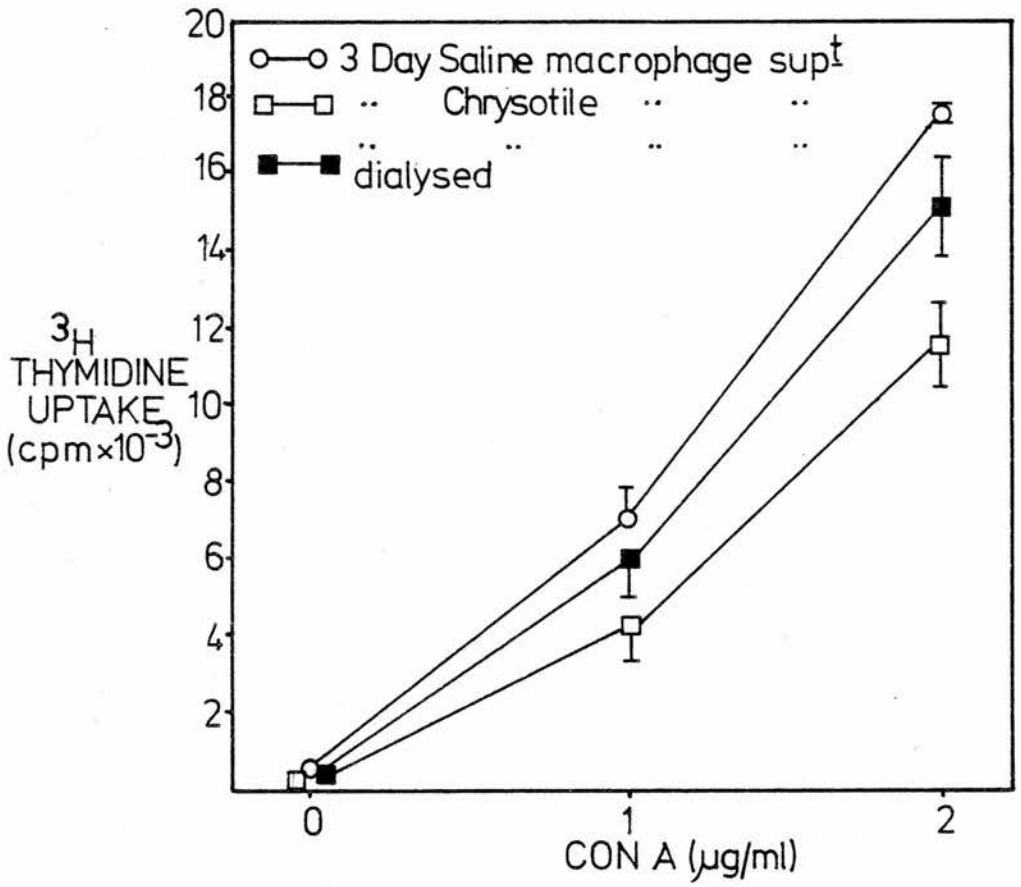


FIGURE 3.56 **Effect of dialysis on chrysotile macrophage supernatant mediated suppression of mitogenesis. Significant reduction in mitogenesis caused by chrysotile supernatant compared to saline supernatant at both 1 µg/ml ($P<0.05$) and 2 µg/ml ($P<0.001$) Con A; significant reduction caused by dialysed supernatant at 2 µg/ml Con A only ($P<0.01$). Symbols denote $\bar{x} \pm$ one standard deviation.** representative exp^{t}.

TABLE 3.21 Effect of treatment with variously induced macrophage supernatants on uptake of tritiated thymidine by fibroblasts.
Significant difference - saline v crocidolite $P<0.01$

TREATMENT	^{3}H THYMIDINE UPTAKE (cpm $\pm$ sd)
Untreated	1079 ± 164
Saline	922 ± 81
Crocidolite	1216 ± 28
Chrysotile	1071 ± 171

pooled data from 3 separate exp$^{ts.}$

TABLE 3.22 Effect of 3 day chrysotile or saline macrophage supernatant on nylon wool column purified splenocyte mitogenesis expressed as mitogenic index *.
Significant differences - chrysotile v saline 1 µg/ml Con A $P<0.05$; 2 µg/ml Con A $P<0.05$.

	CON A µg/ml	
	1	2
Saline	0.88 ± 0.10*	0.98 ± 0.05
Chrysotile	0.70 ± 0.10	0.89 ± 0.02

* Mitogen index derived as in Table 3.20
pooled data from 2 separate exp$^{ts.}$

than these produced by saline macrophage supernatants ($P < 0.05$ for both 1 and 2 μg/ml Con A).

3.5.11 Effect of serum from 3 day chrysotile injected mice on mitogenesis

To determine whether the factor suppressing mitogenesis, released by asbestos induced macrophages, was present in the serum, mice were killed 3 days after saline, crocidolite or chrysotile injection and the serum collected. Initially serum was used at 10% in mitogenesis assays, but as shown in Fig. 3.57, at this level there was almost complete inhibition of thymidine uptake. When the same serum was present as 1%, inhibition was much less but there was no evidence of increased inhibitory activity in the sera from asbestos injected mice.

3.5.12 Effects of asbestos on mitogenesis

It was possible that asbestos itself could have some direct effects on lymphocytes which could affect their function; it was also possible that very small fibres, if exocytosed by macrophages during generation of supernatants, might not be spun out and could thus contribute to the properties of the supernatant. Asbestos was therefore tested in thymocyte mitogenesis assays. It is clear from Fig. 3.58 that both crocidolite and chrysotile were inhibitory to background thymidine incorporation by thymocytes (0 μg/ml Con A) with crocidolite being significantly inhibitory above 0.01μg/well ($P < 0.02$). With chrysotile only 0.001 μg/well ($P < 0.02$) and 10 μg/well ($P < 0.05$) were significantly inhibitory to thymidine uptake in the absence of Con A. At 2 μg/ml Con A crocidolite had no effect on mitogenesis across the 5 orders of magnitude of dose used; chrysotile, however, was significantly stimulatory to thymidine uptake at all doses ($P < 0.05$ to $P < 0.002$). With 4 μg/ml Con A again there was no significant difference in thymidine uptake between control thymocytes and thymocytes incubated with doses of crocidolite across 5 orders of magnitude; chrysotile was again significantly stimulatory at all doses ($P < 0.05$ to $P < 0.01$).

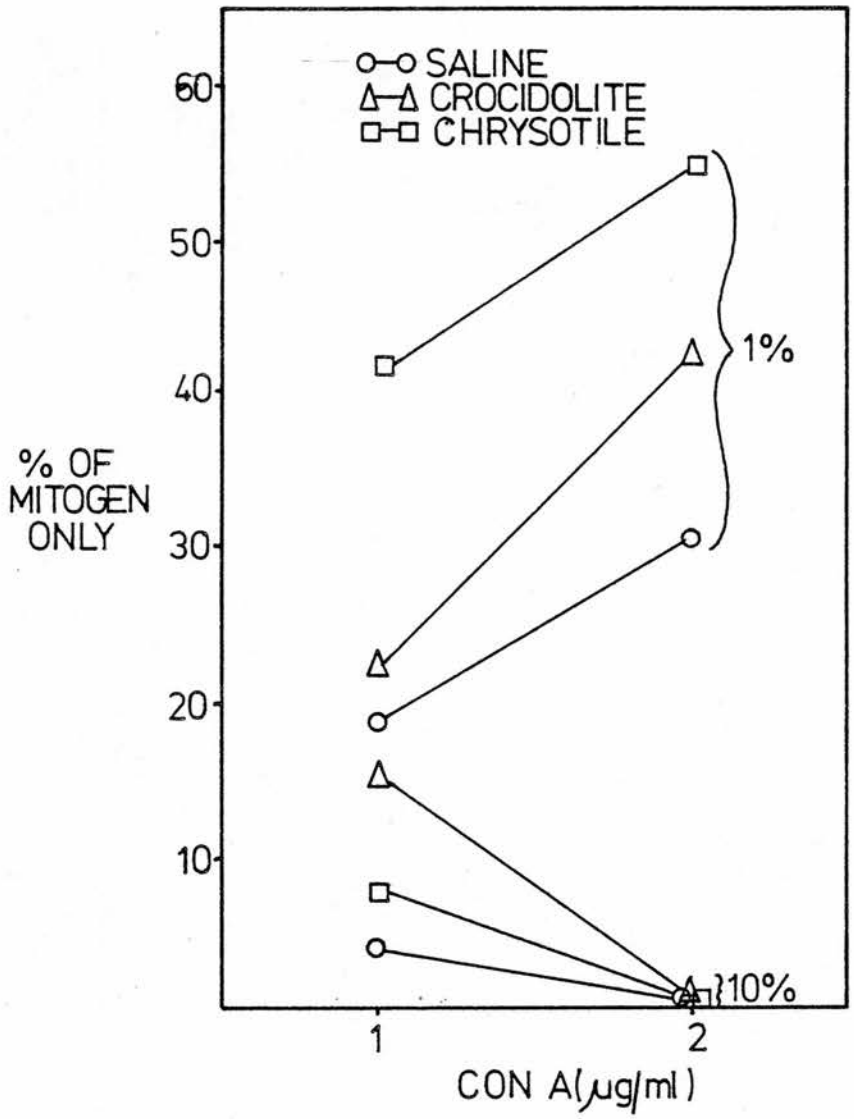


FIGURE 3.57 **Effect on Con A mitogenesis of serum from saline, crocidolite or chrysotile injected mice as 1 and 10% of incubation volume.**

$\bar{x}$ of 2 mice for each condition

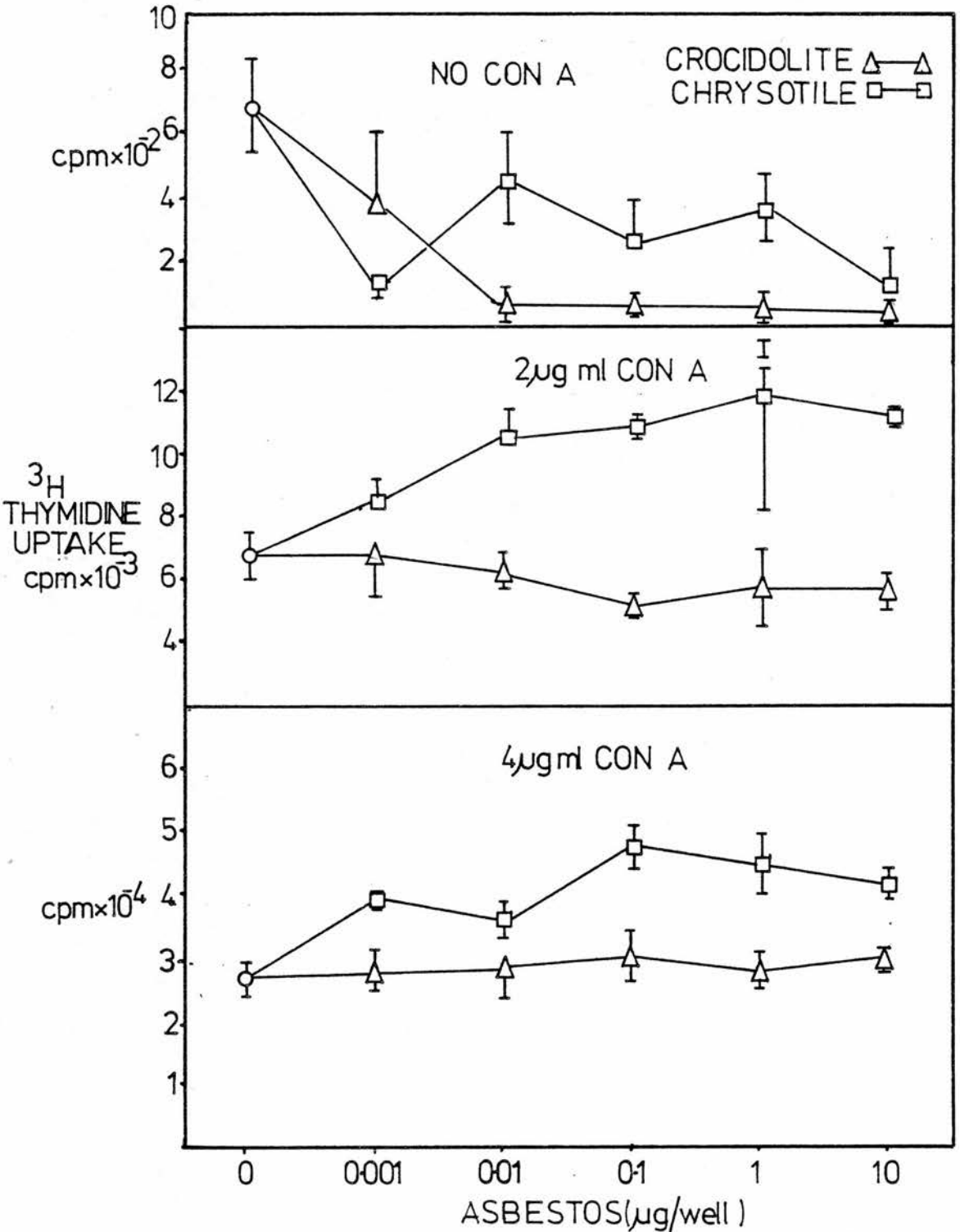


FIGURE 3.58 Effect of crocidolite and chrysotile on Con A mitogenesis of thymocytes.
Symbols denote $\bar{x} \pm$ one standard deviation (repr. expt.)
Significant differences from no asbestos:- no Con A - crocidolite above 0.01 μg/ml/well was inhibitory ($P<0.02$); chrysotile was inhibitory at 0.001 μg/well ($P<0.02$) and 10 μg/well ($P<0.05$).
2 ug/ml Con A - 0.001-10 μg/well chrysotile - significant stimulation ($P<0.05$-$P<0.002$).
4 μg/ml Con A - 0.001-10 μg/well chrysotile - significant stimulation ($P<0.05$-P 0.01).

3.5.13 Effect of chrysotile leachate on thymocyte mitogenesis

Table 3.23 shows that 50% chrysotile leached medium, present during the mitogenesis assay, had no significant effect on thymidine uptake by the thymocytes.

3.6 Attempts to detect differences in surface antigenicity between saline and crocidolite induced macrophages using anti-sera raised against PEC

Anti-sera were raised in rabbits against 6 day saline and crocidolite induced PEC and experiments were carried out to determine whether these anti-sera, suitably absorbed, could be used to detect differences in their respective macrophage components with regard to surface antigenicity using a $[^{125}I]$ Protein A binding assay.

3.6.1 Binding to thymocytes and control PEC following absorption of anti-sera

Anti-serum 151 was raised against 6 day saline induced PEC and anti-serum 152 was raised against 6 day crocidolite induced PEC. All anti-sera used were obtained 2 weeks after the end of the immunisation schedule (151/2; 152/2).

Non-specific anti-mouse activity was removed by absorption until a target binding index of one was obtained:-

$$\text{binding index} = \frac{[^{125}I] \text{ Protein A (cpm) bound with immune serum at dilution n}}{[^{125}I] \text{ Protein A (cpm) bound with prebleed serum at dilution n}}$$

(i) Prebleeds Absorbing prebleed sera with 10^6, 10^7 or 10^8 thymocytes/ 100 µl made no difference to binding which remained close to background binding obtained by substituting PBS for the anti-serum step (Fig. 3.59) This result was expected since the prebleeds were, in effect, normal rabbit serum.

(ii) Anti-sera As shown in Fig. 3.60 absorbing the anti-sera with 10^6, 10^7 or 10^8 thymocytes /100 µl resulted in a dose dependent decrease in anti-

TABLE 3.23 **Effect of chrysotile leachate on thymocyte mitogenesis**
(cpm $\bar{x}^{+}$ sd of 3 separate exp^{ts}.)
No significant differences.

	CON A (μg/ml)		
	0	1	2
Mitogen only	769^{+}219	4452^{+}538	12,388^{+}2094
+50% Chrysotile Leachate	553^{+}60	4475^{+}758	14,109^{+}822

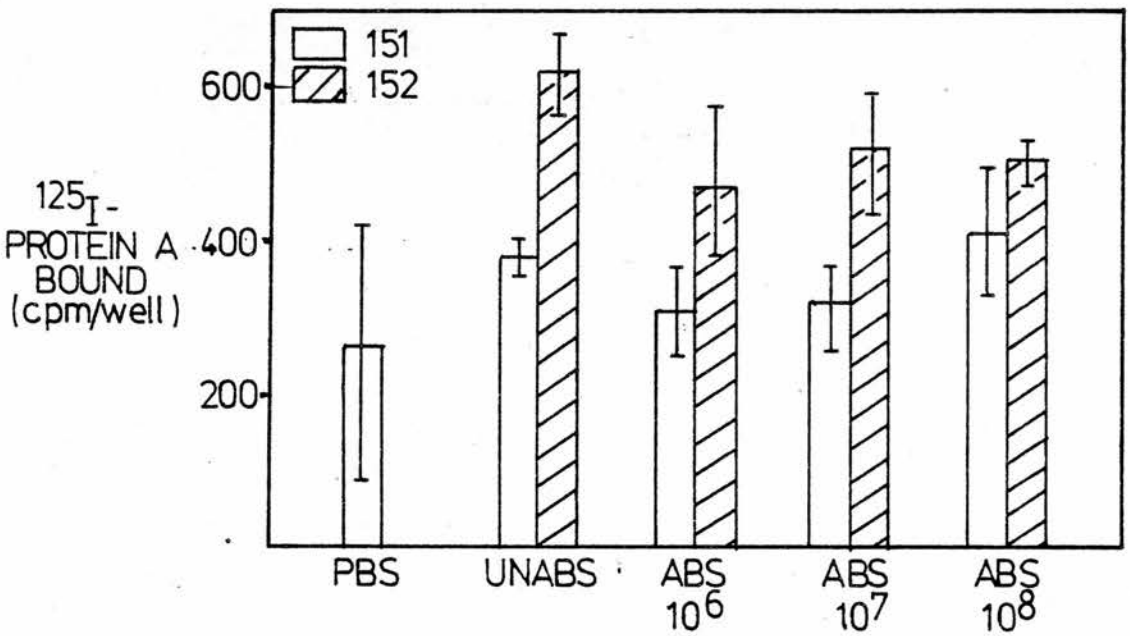


FIGURE 3.59 **Effect of absorption of prebleeds with 10^6-10^8 thymocytes on binding to thymocytes** ($\bar{x}$±sd of triplicate wells: repr exp^t.)

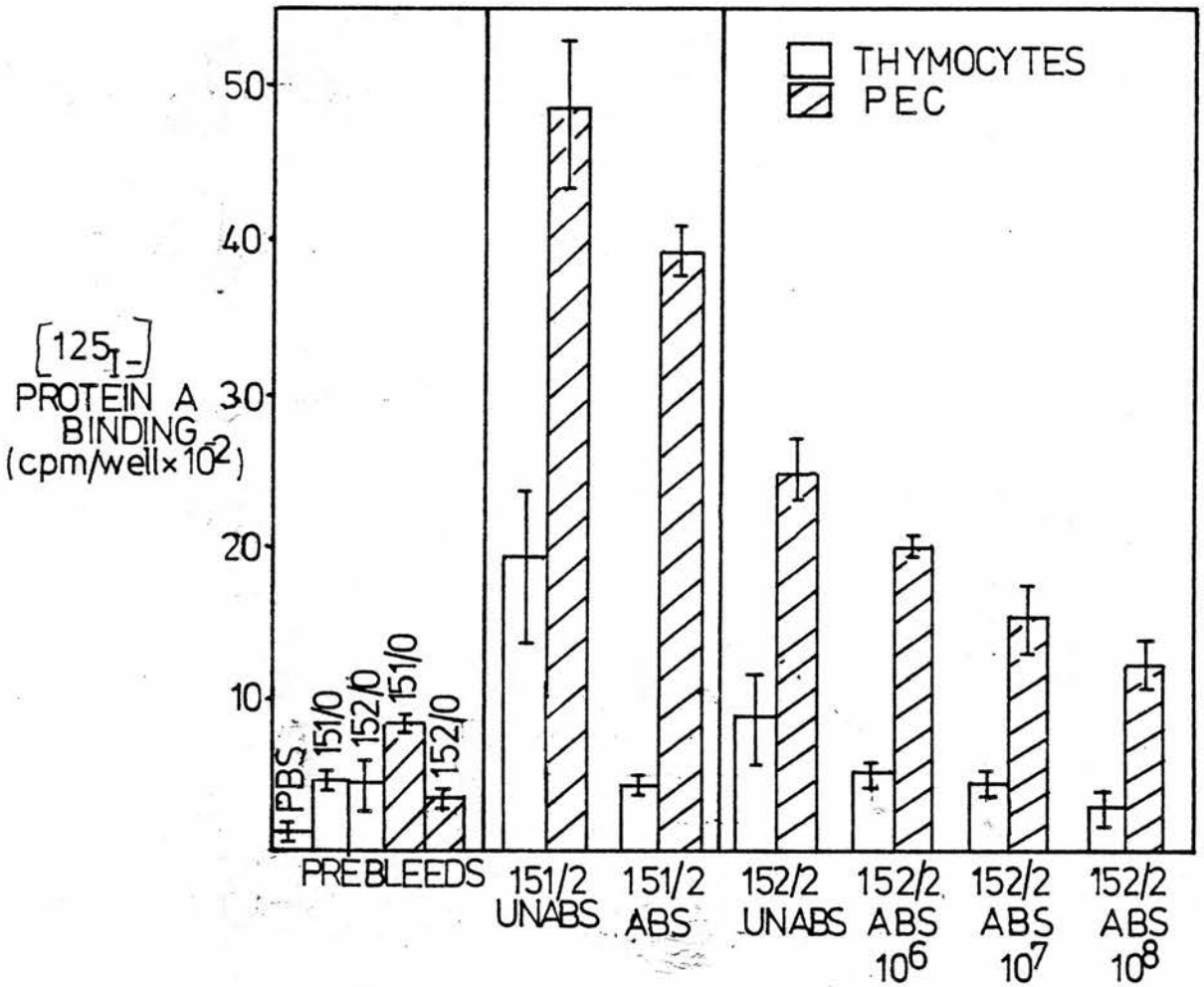


FIGURE 3.60 **Effect of absorbing anti-sera with thymocytes on binding to thymocytes and whole PEC.***

TABLE 3.24 Binding indices of 151 and 152, absorbed with 10^8 thymocytes/ 100 µl, against thymocyte and PEC targets.

SERUM	THYMOCYTES	BINDING INDEX	PEC	BINDING INDEX
151/0 Pre-bleed	412±86*	1.1	772±52	5.1
151/2	454±40		3906±136	
152/0 Pre-bleed	294-73	1.2	294-42	4.1
152/2	213-98		1196-146	

* $\bar{x}\pm$ standard deviation (triplicate wells: repr. expt.)

serum binding. With 10^8 thymocytes/100 µl all anti-thymocyte activity was removed as shown by binding indices of 1.1 for 151 and 1.2 for 152 (Table 3.24). After absorbing out the anti-thymocyte activity anti-PEC activity remained. This was greater with 151; 152 had only $\frac{1}{3}$ of the activity of 151 but, due to increased binding of the 151 prebleed the binding indices of 151 and 152 to PEC were similar (Table 3.24).

3.6.2 Binding to 6 day saline and 6 day crocidolite induced PEC of absorbed and unabsorbed anti-sera

As shown in Fig. 3.61, 151 bound in greater amounts to both saline and crocidolite PEC than 152. It is also evident that absorbed 151 bound significantly ($P < 0.001$) more to saline PEC than to crocidolite PEC. There was no significant difference in the binding of 152 to either saline or crocidolite PEC. Absorption with 10^8 thymocytes/100 µl caused significant ($P < 0.01$ to $P < 0.001$, see Fig. 3.61 for details) decreases of $\frac{1}{3}$ to $\frac{1}{2}$ in the binding of both anti-sera to both targets; this decrease represented the shared antigen component between thymocytes and PEC.

3.6.3 Binding of anti-sera to whole PEC and to the macrophage fraction

As shown in Fig. 3.62 both anti-sera bound less to adherent than to whole PEC and the reduction in binding ranged from 33% to 53% ($P < 0.05$ to $P < 0.01$; 152/2 v 6 day crocidolite not significant). Anti-serum 151 bound significantly ($P < 0.001$) more to saline macrophages than to crocidolite macrophages but there was no significant difference in the binding of anti-serum 152 to either type of macrophage.

3.6.4 Kinetics of binding of the anti-sera

The previous experiments had established that activity was present, in both anti-sera, against whole PEC and against the macrophage component. All these experiments were carried out at a nominal anti-serum dilution of 1/50 and in order to actually compare the anti-sera in terms of their

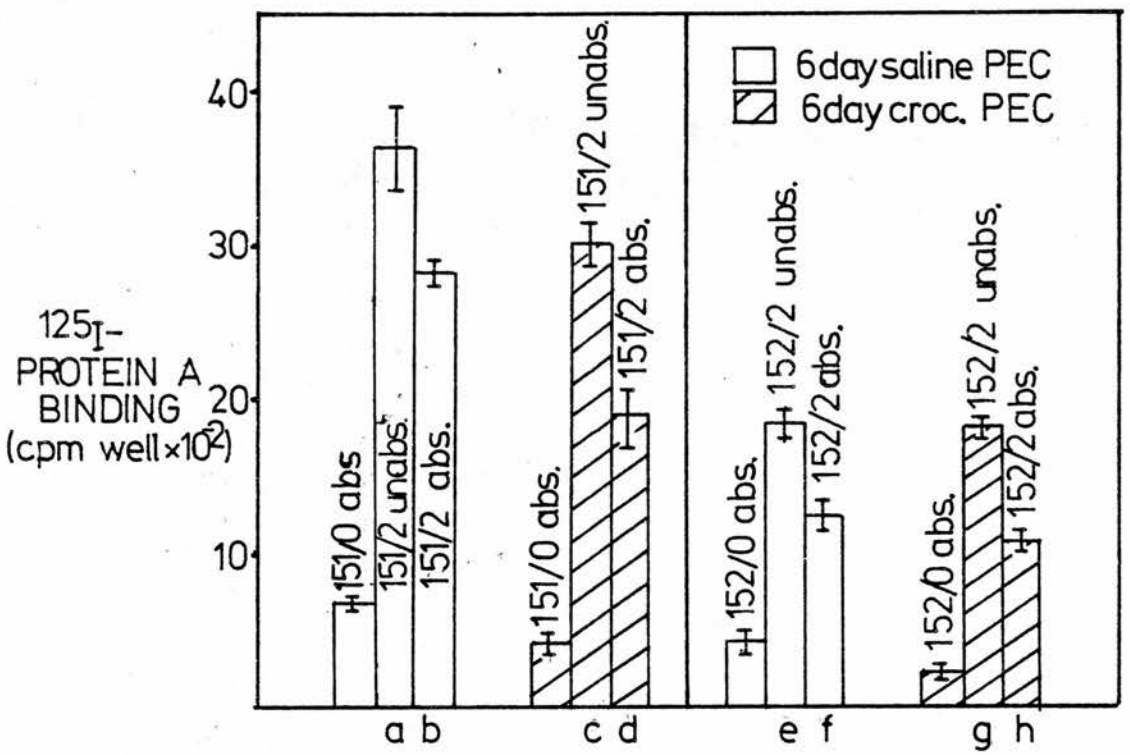


FIGURE 3.61 **Binding of 151 and 152 to 6 day saline and 6 day crocidolite induced whole PEC.**
Significant differences - bvd $P<0.001$; avb $P<0.01$; cvd $P<0.001$; evf $P<0.001$; gvh $P<0.001$.
Columns and bars denote $\bar{x} \pm$ one standard deviation.
(triplicate wells: repr. exp^t)

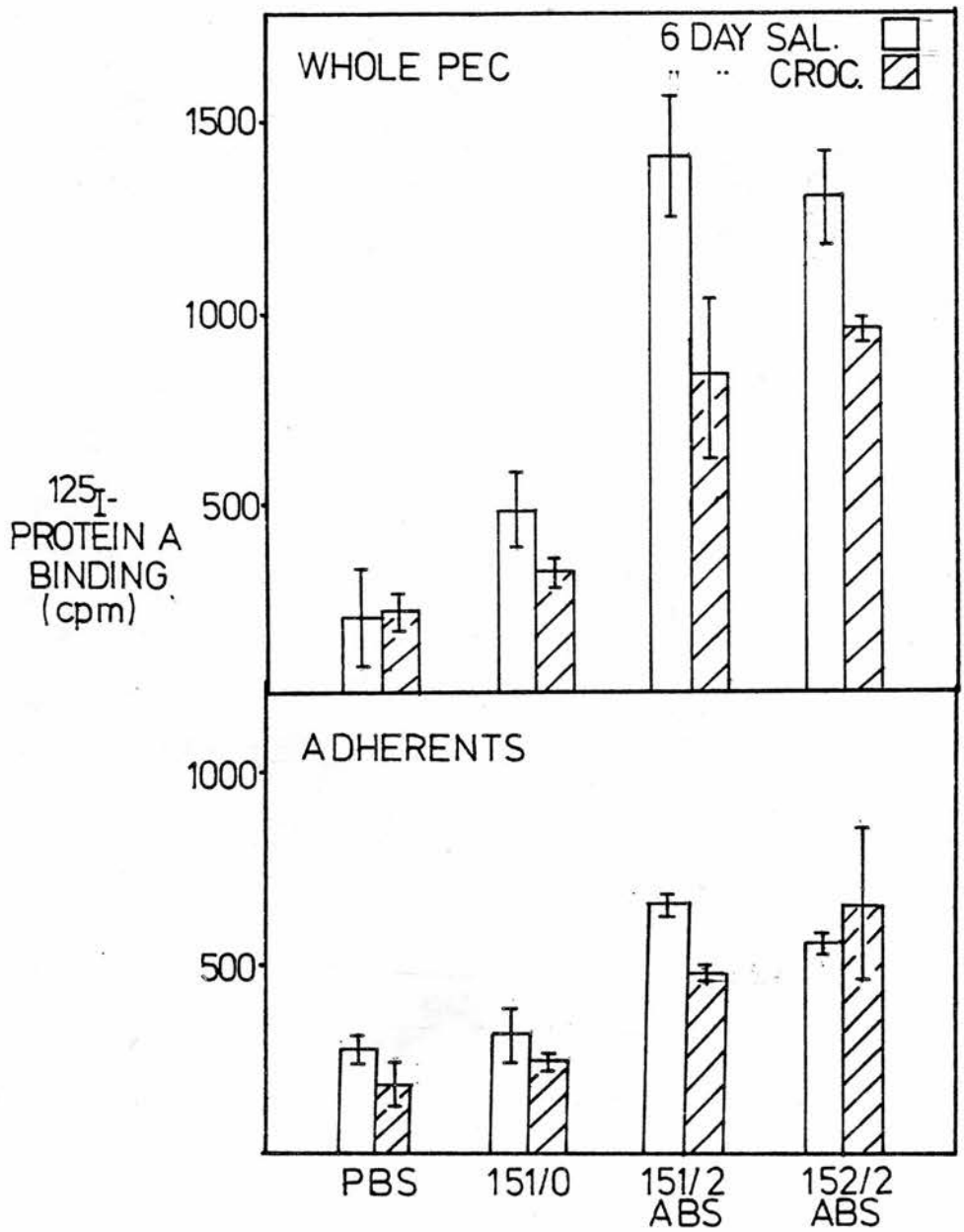


FIGURE 3.62 **Binding of anti-sera to total and adherent fraction of 6 day saline and 6 day crocidolite induced PEC. Significant difference - 151/2 saline v crocidolite** $P<0.001$ ($\bar{x}$±sd of triplicate wells: repr. exp^{t}.).

binding activity it had to be confirmed that saturation binding conditions pertained at this dilution. As shown in Fig. 3.63 saturation binding was not reached at a dilution of 1/10 with any of the anti-serum directed against either of the target adherent cells. It was apparent that the greatest activity in both anti-sera was against the saline induced macrophages.

In a further, final, attempt to obtain saturation binding the number of target cells was reduced but as shown in Fig. 3.64 reducing the number of targets and using anti-sera at 1/10 dilution failed to result in significant binding over and above background (1/10 prebleed).

Attempts to detect differences in the surface antigenicity of 6 day saline and crocidolite induced macrophages using the $\left[^{125}I\right]$ Protein A assay and anti-serum raised in rabbits, was abandoned due to the low specific "anti-macrophage surface" activity in the anti-sera.

3.6.5 Immunofluorescence

Although the two anti-sera could not be used to quantitate differences between control and asbestos induced macrophages, immunofluorescence was carried out using the two anti-sera to determine whether qualitative differences could be detected between them in terms of the staining pattern.

Fig. 3.65 (upper) shows 6 day saline macrophages incubated with pre-bleed serum (151/0) then FITC-GαR and no membrane staining is seen; a similar appearance was produced with 152/0. Fig. 3.65 (lower) shows 6 day crocidolite induced macrophages stained with 151/2 while Fig. 3.66 (upper) shows the same cells stained with 152/2; a similar appearance with ring staining is evident with both anti-sera. Using both anti-sera against 6 day saline induced macrophages the same appearance was obtained but more cells remained unspread as in Fig. 3.66 (lower).

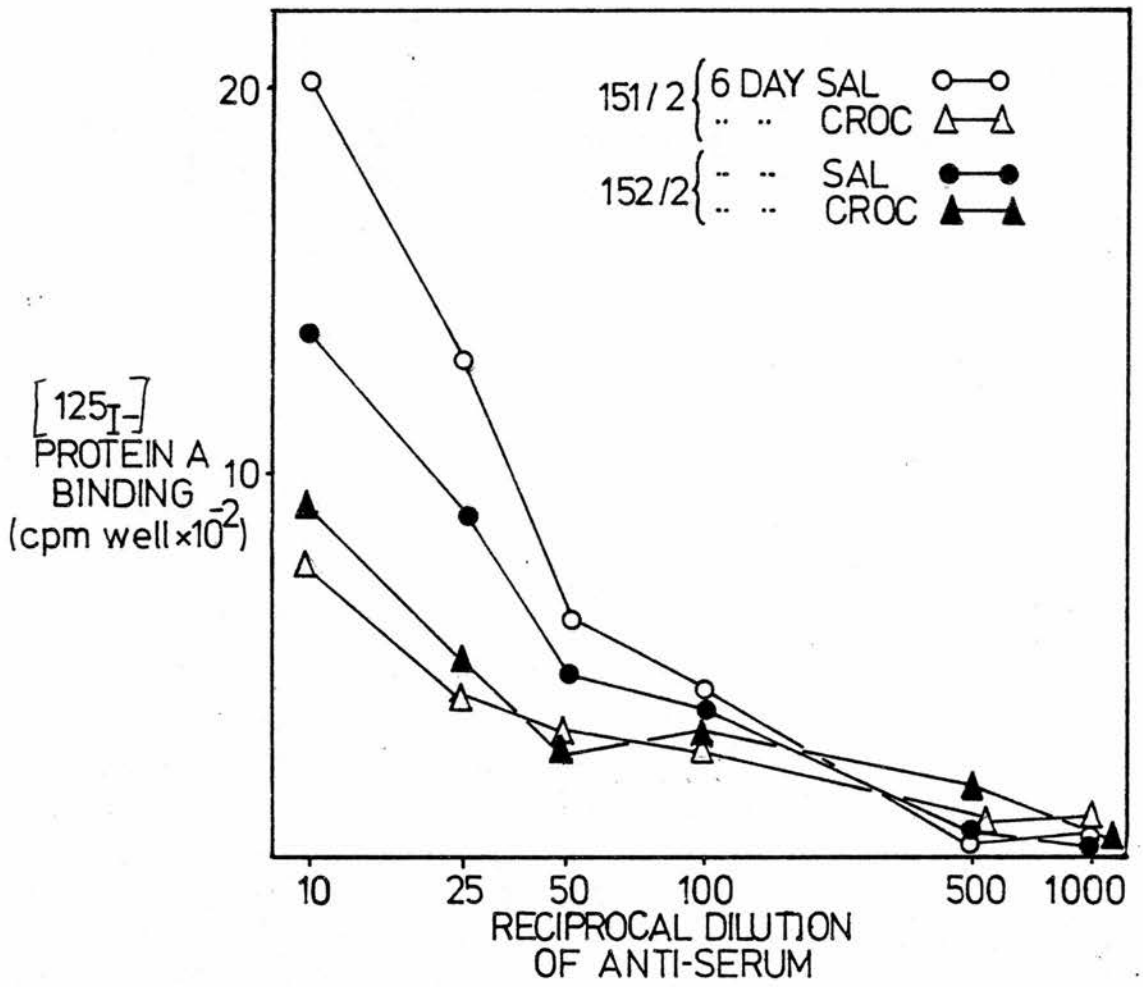


FIGURE 3.63 **Effect of increasing the concentration of anti-sera used in the assay on binding to macrophage targets.** ($\bar{x}$ of duplicate wells)

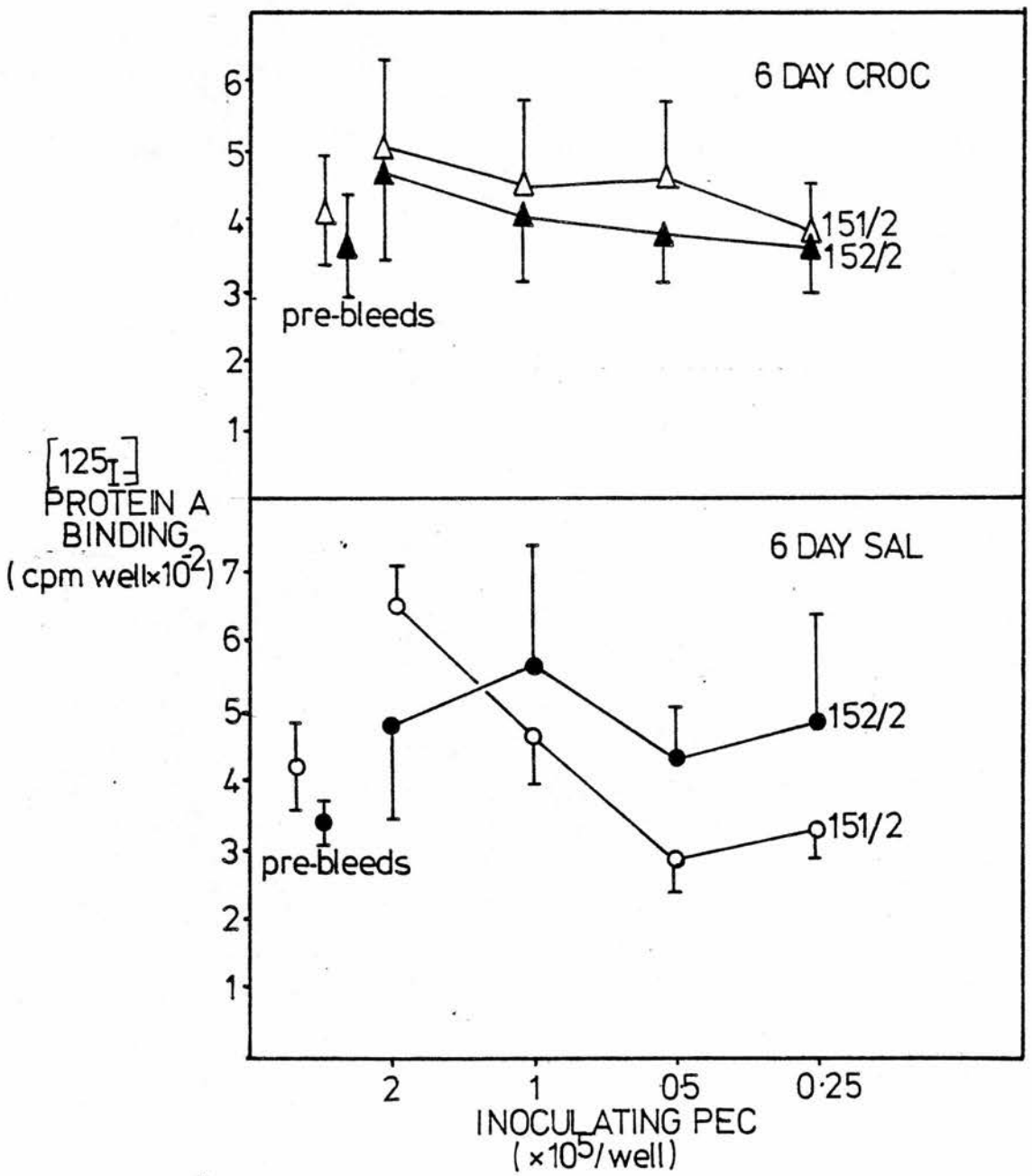


FIGURE 3.64 **Effect of decreasing the target cell concentration, on anti-serum binding.**

(x±sd of triplicate wells: repr exp^{t})

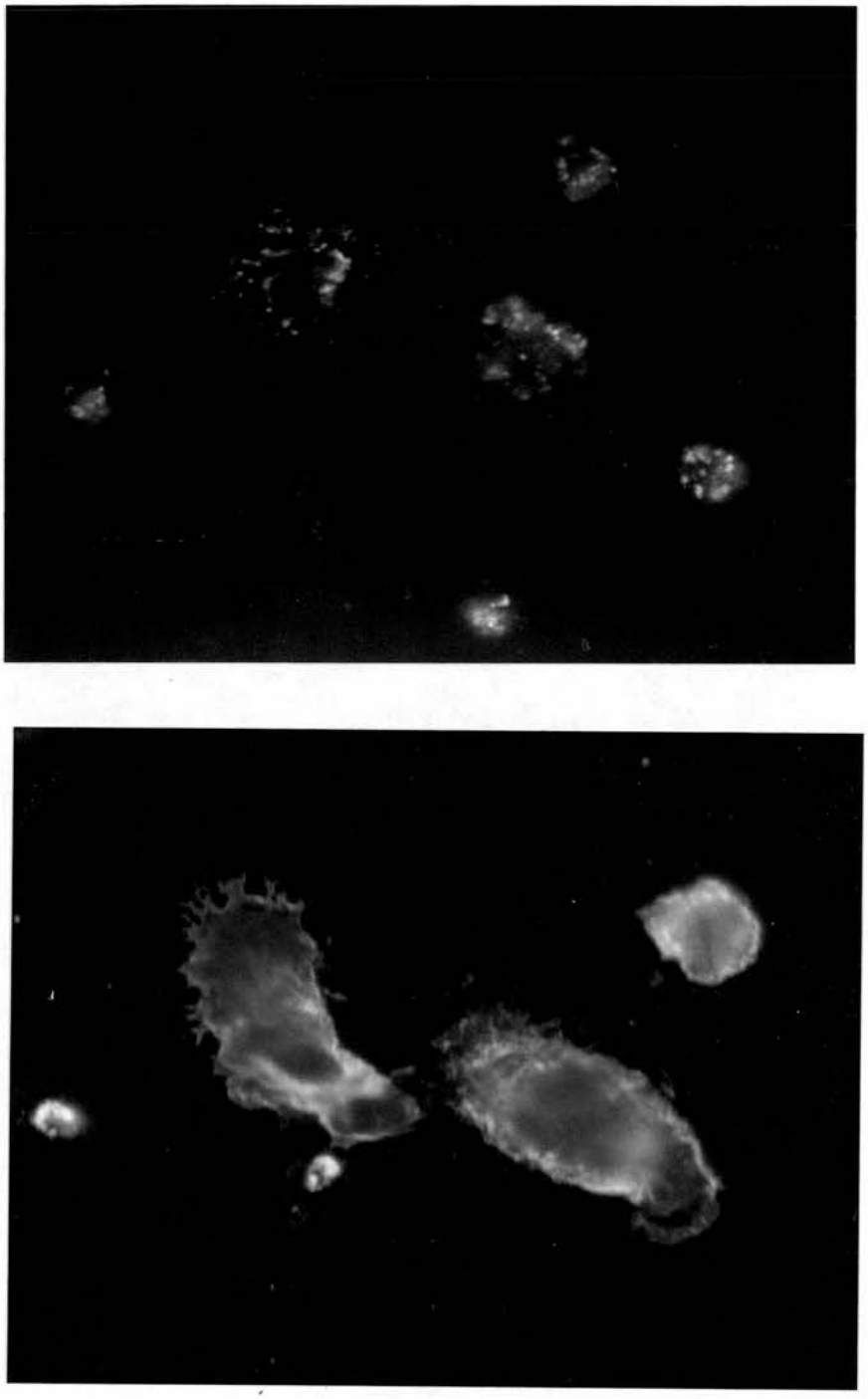


FIGURE 3.65 Upper - 6 day saline induced macrophages pre-incubated with prebleed serum then FITC-G∝R.
Lower - 6 day crocidolite induced macrophages pre-incubated with 151/2 (anti-serum to saline induced macrophages) then FITC-G∝R.
Mag. x 600.

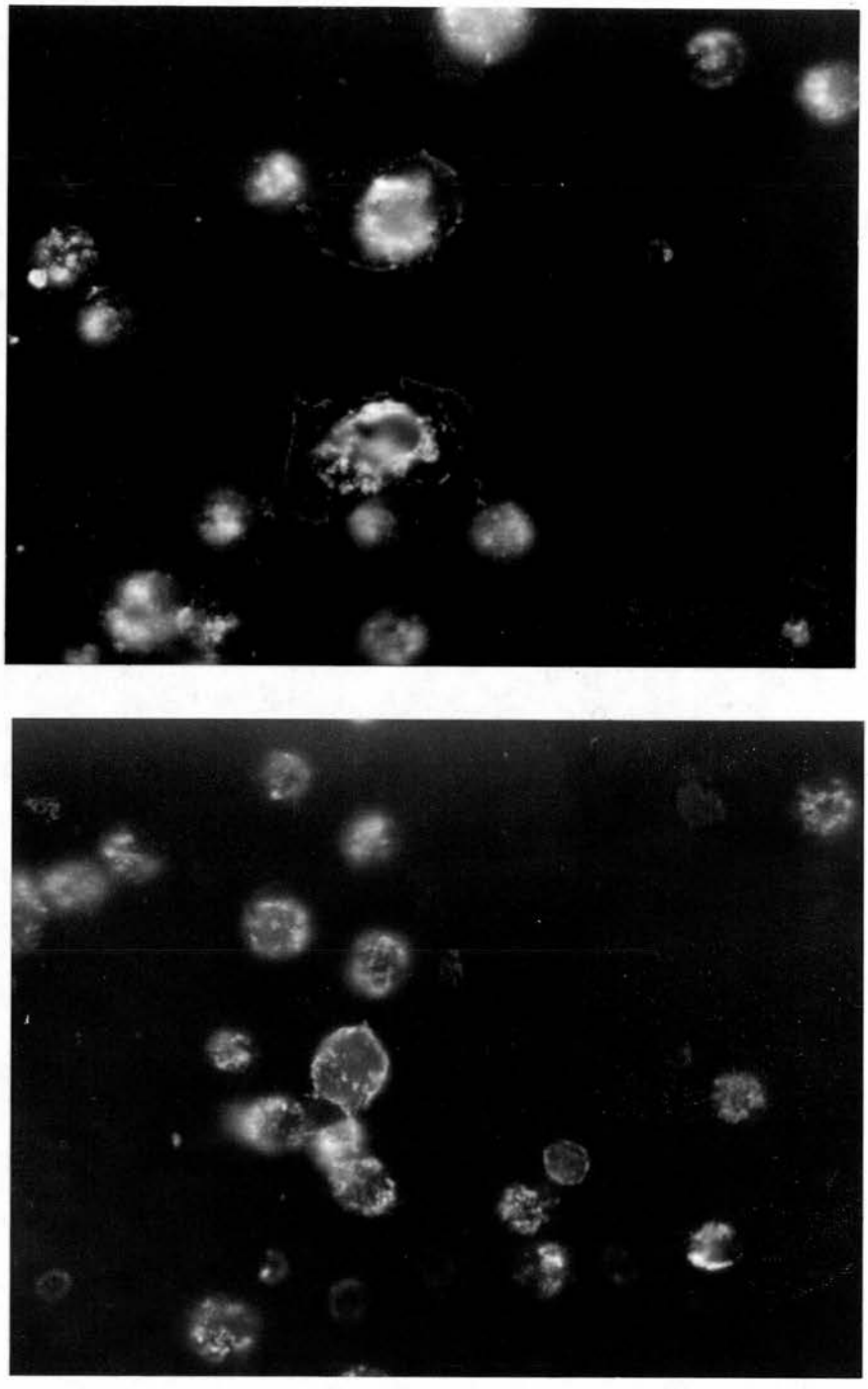


FIGURE 3.66 Upper - 6 day crocidolite induced macrophages pre-incubated with 152/2 (anti-serum to crocidolite induced macrophages) then FITC-G∝R. Lower - 6 day saline induced macrophages pre-incubated with 152/2 then FITC-G∝R.

Mag. x 600.

In blocking experiments macrophages were exposed to one anti-serum followed by unlabelled GαR then the second anti-serum and finally FITC-GαR. Blocking with either anti-serum resulted in considerable reduction in membrane fluorescence but occasional cells retained bright ring fluorescence as shown in Fig. 3.67 (upper) (6 day crocidolite macrophages blocked with 151 then stained 152) and Fig. 3.67 (lower) (6 day saline macrophages blocked with 151 then stained for 152). These ring staining cells which represented cells recognised by the second anti-serum but not by the first (blocking) anti-serum were quantified as given in Table 3.25. Each anti-serum appeared to recognise membrane elements not recognised by the other, but in general 152 appeared to contain more activity against both 6 day saline and 6 day crocidolite macrophages than 151.

3.6.6 Complement mediated lysis

Using complement mediated lysis to assay for the cytotoxic antibody activity of the 2 anti-sera revealed no real differences. As shown in Table 3.26 cytotoxicity in all cases was between 11 and 21% while the various controls ranged from 0-10.6% cytotoxicity.

3.7 In vivo autoimmune deposition of immunoglobulin on asbestos induced macrophages

In these experiments attempts were made, using anti-serum to mouse immunoglobulin and $\left[^{125}I\right]$ Protein A, to detect autoimmune deposition of antibody on the surface of freshly harvested asbestos induced macrophages and controls, 6 days, 14 days and 294 days after crocidolite injection.

3.7.1 Effects due to size and Fc receptor binding

Prebleed 151/0 and anti-serum 151/2 (unabsorbed) were used to detect any differences between the two macrophage populations with regard to Fc receptor and size respectively.

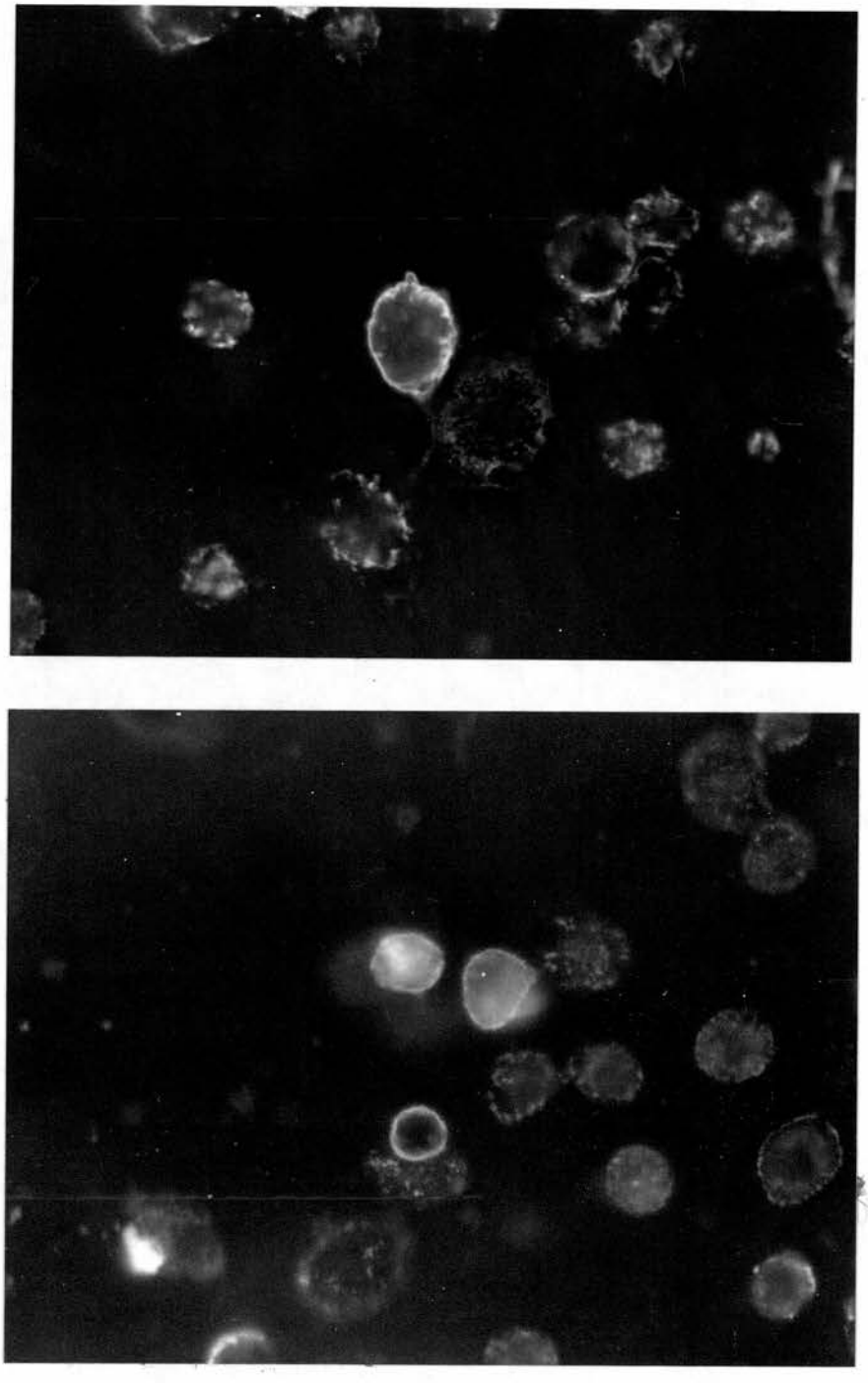


FIGURE 3.67 Figures represent preparations from "blocking experiments (see text for details).
Upper - 6 day crocidolite macrophages blocked with 151 then stained for 152.
Lower - 6 day saline macrophages blocked with 151 then stained for 152.

Mag. x 600

TABLE 3.25 Percentage of macrophages showing ring fluorescence with either anti-serum and FITC-GαR following blocking with the other anti-serum plus unlabelled GαR. ($\bar{x} \pm sd$)

	BLOCKED WITH 151 STAINED FOR 152	BLOCKED WITH 152 STAINED FOR 151
6 day saline	29.5±4.8	11.3±2.0
6 day chrysotile	33.1±6.9	9.9±0.4

*

TABLE 3.26 Complement mediated cytotoxicity of anti-sera 151 and 152 to 6 day saline and 6 day crocidolite macrophages, plus controls.

TREATMENT	% CYTOTOXICITY	
	6 DAY SALINE	6 DAY CROCIDOLITE
PBS	4.0	10.6
151/2 + Heat Inactivated Complement	9.6	4.0
152/2 + Heat Inactivated Complement	2.7	1.0
PBS + Complement	7.9	0.0
151/0 + Complement	0.5	4.2
152/0 + Complement	1.1	3.3
151/2 + Complement	11.4±1.8	20.8±7.7
152/2 + Complement	15.2±2.5	13.6±3.2
Anti-thymocyte + Complement	Thymocyte Targets 71.3	

* pooled data 2-4 exp^{ts}

(i) Fc receptor Prebleed serum was used to detect Fc binding of antibody. Table 3.27 reveals no significant difference in binding of 151/0 to saline macrophages at all time points or crocidolite macrophages at all time points.

(ii) Cell surface area As a general index of size unabsorbed 151/2 (raised against 6 day saline PEC) was used and no significant difference in binding was found between saline and crocidolite induced macrophages comparing all time points (Table 3.28). This suggests that there was no gross difference in surface area between saline and asbestos induced macrophages at least with regard to normal macrophage antigens available for recognition by 151/2.

3.7.2 Binding of rabbit anti-mouse immunoglobulin

Expressing the binding of anti-mouse immunoglobulin in terms of binding of prebleed serum at the same concentration (1/50) yielded binding indices for each experiment and these are shown in Table 3.28. There is clearly no increase in macrophage cytophilic immunoglobulin as recognised by the rabbit anti-mouse immunoglobulin in crocidolite induced macrophages compared to saline induced macrophages at any time point. Pooling the results from all the time points yielded a figure for all experiments (Table 3.28) which showed no significant difference between saline and crocidolite induced macrophages.

3.8 Long term effects of intraperitoneal asbestos in mice

At the onset of this study mice were injected ip with asbestos and left to live out their life span to determine the long term effects of asbestos in the model.

3.8.1 Fibrogenic effects

It was evident that all mice killed a long time after asbestos injection had a considerable degree of fibrosis in the peritoneal

TABLE 3.27 Binding of anti-serum 151/2 and prebleed 151/0 (normal rabbit serum) to saline and asbestos induced macrophages. Figures represent mean $\pm$ standard deviation of all experiments, ie. 2 at 6 days, 2 at 14 days and 2 at 42 weeks.

	[^{125}I] PROTEIN A BOUND/ WELL ($\bar{x} \pm$ sd cpm)	
	SALINE	CROCIDOLITE
PBS	93 ± 35	114 ± 23
151/0 (NRS)	149 ± 68	180 ± 46
151/2 (Unabsorbed)	808 ± 394	967 ± 368

TABLE 3.28 Binding indices of anti-mouse immunoglobulins to saline and asbestos induced macrophages various times after injection of saline or asbestos.

$$\text{Binding index} = \frac{\text{Binding of Protein A (cpm) after treatment with rabbit anti-mouse immunoglobulin}}{\text{Binding of Protein A (cpm) after treatment with normal rabbit serum}}$$

	6 DAYS	14 DAYS	42 WEEKS	ALL EXPS.
Saline	1.6 *	2.1	1.6	1.8 ± 0.3
Crocidolite	1.7	1.8	1.8	1.8 ± 0.1

* Each figure represents the mean of two experiments at that time point.

cavity. This was present as nodules (granulomas) and sheets of fibrous tissue often binding portions of the peritoneal viscera together as shown in Fig. 3.1. The principal cell type in these lesions was the fibroblast although giant cells and macrophages were often seen. Fig. 3.68 (upper) shows an area of fibrosis from a mouse which had been injected with crocidolite 15 months previously. The centre of the lesion is largely acellular collagen with a few giant cells while the periphery consists of fibroblasts and macrophages; many fibres of crocidolite are visible lying within the fibrous tissue and between the cells. Crocidolite fibres are more clearly seen in the higher power view shown in Fig. 3.68 (lower). Some asbestos fibres lying within areas of fibrosis could be seen, at higher power, to have refractile "beads" on them and on staining with Perle's reaction for iron pigment these "beads" stained positively revealing them to be asbestos (ferruginous) bodies (Fig. 3.69); it was notable that only a minority of fibres had become coated as is evident from Fig. 3.69.

Mice injected at the same time as these mice, but which received saline or latex, never showed any evidence of fibrosis in any of the sections examined.

Five mice kept from 13-17 months after intraperitoneal injection of 2.5 mg of C. parvum showed no evidence of fibrosis in the peritoneal cavity at autopsy or in sections of the peritoneal viscera.

3.8.2 Carcinogenic effects

3.8.2.1 Ascites and malignant cell lines

The major long term effect of ip asbestos in CBA/Ca mice was the development of peritoneal ascites which led rapidly to death if undetected. The typical appearance of a mouse killed after developing ascites is shown in Fig. 3.70. As shown in Table 3.29, 17 mice in all developed ascites following asbestos injection with a mean time to ascites

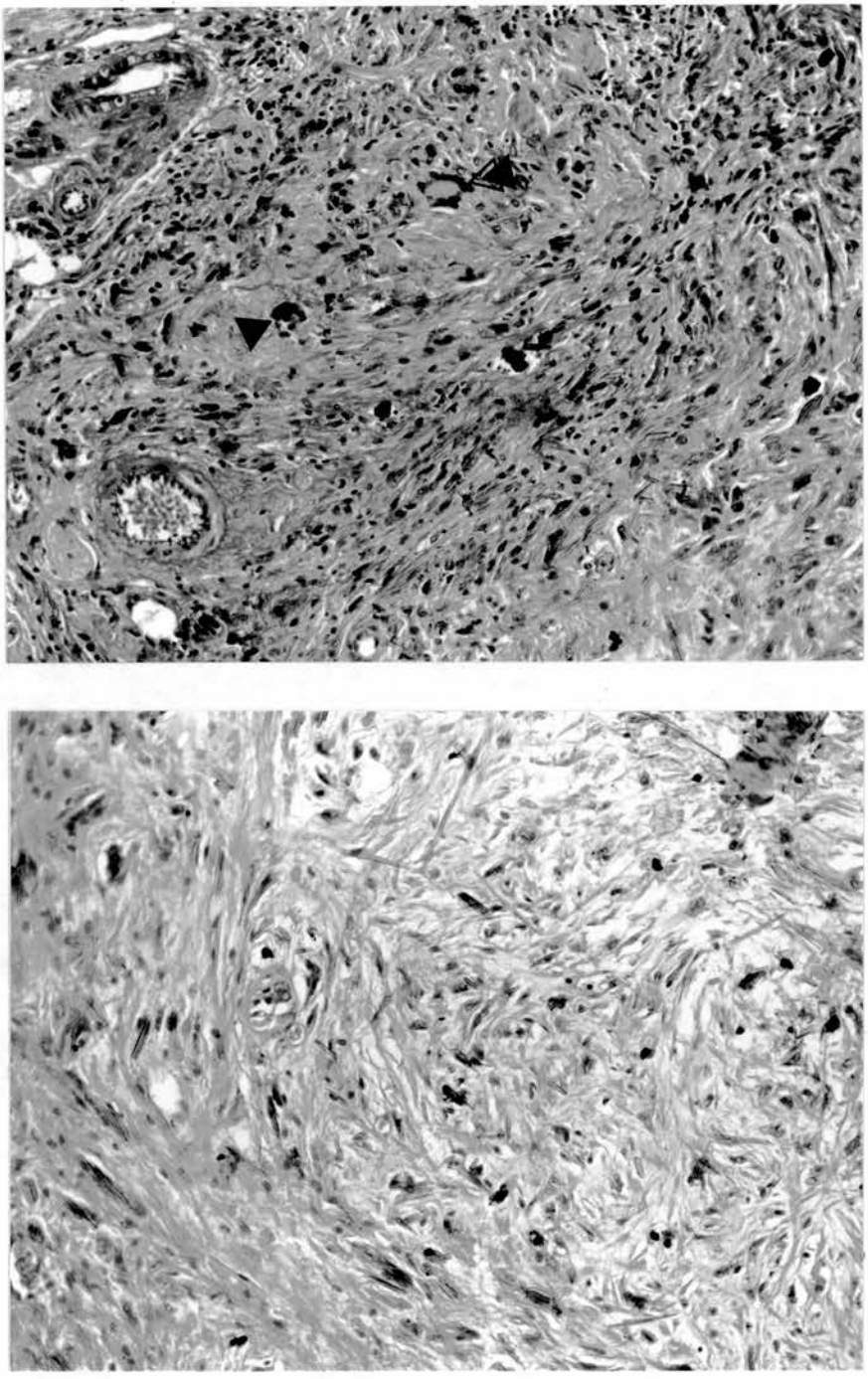


FIGURE 3.68 Fibrous lesion in the peritoneal cavity of a mouse injected with crocidolite 15 months before killing.
Upper - Giant cells are visible in the centre of the lesion (arrows) which is essentially acellular collagen.
Lower - higher power view of adjacent field to show crocidolite fibres embedded in fibrous matrix.
Mag. - upper x 150; lower x 300.

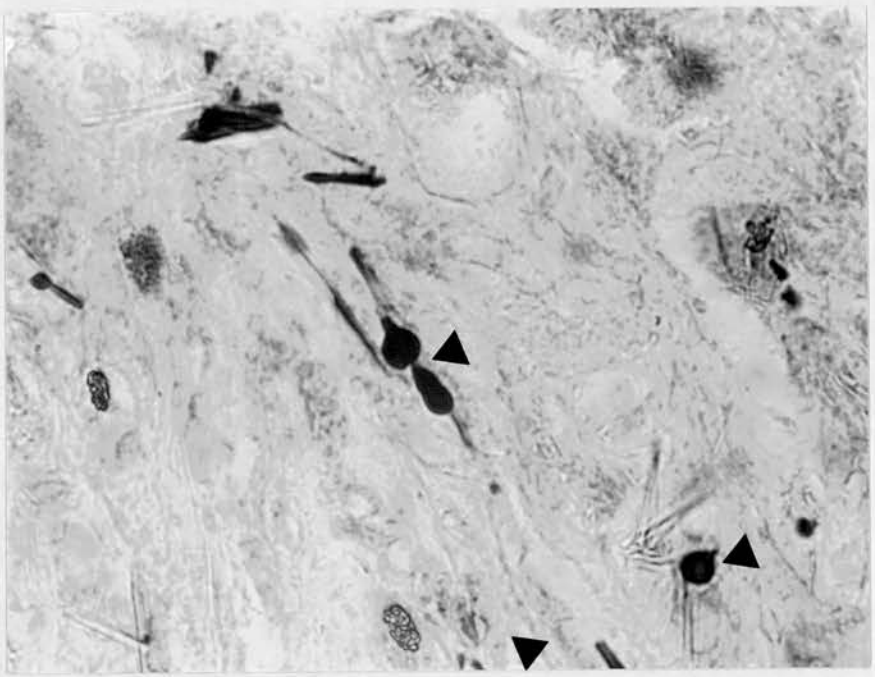


FIGURE 3.69 Asbestos fibres present in a fibrous lesion from the peritoneal cavity of a mouse killed after injection of crocidolite. Two of the fibres have been coated (arrows) but many are uncoated. Perle's reaction. Mag. x 750

FIGURE 3.70 Comparison of control mouse (top) with mouse having abdominal distension following long term crocidolite injection. At autopsy the lower mouse was found to have peritoneal ascites.

TABLE 3.29 Relationship of ip asbestos type and dose with time to develop of peritoneal ascites.

ASBESTOS TYPE	DOSE (ip)	MEAN TIME TO ASCITES DEVELOPMENT	NUMBER OF ANIMALS
Crocidolite	2.5	462±21	4
Crocidolite	15.0	429±75	5
Chrysotile	15.0	546±16	5
Amosite	5.0	476±10	3

development of 478 $\pm$ 49 days. The reason that several different doses of asbestos were used (Table 3.29) as well as amosite (brown) asbestos which was not otherwise used in this study, was that these animals were injected very early in the study. It was not known at this stage exactly which asbestos types or doses would be used but experience at the Institute of Occupational Medicine suggested that it would take up to 1½ years for tumours to develop so a range of asbestos types and doses was used. It was gratifying to find that 2.5 mg of crocidolite (the dose finally chosen for these experiments) did cause ascites development.

The ascites so produced was never bloodstained but was a cell rich, straw coloured fluid 2-5 ml in volume. The cellular content, when measured, was approximately $3x10^6$ cells/ml of cells which were $>$ 90% macrophages on morphological grounds with a small percentage of lymphocytes. No obvious malignant or abnormal cells were seen nor any macroscopic or microscopic evidence of tumour deposits in the peritoneal cavity. Notwithstanding these findings, on two separate occasions ascites cells were maintained in long term culture in cRPMI and piled up colonies of cells developed in the flasks after 5-6 weeks (Fig. 3.71). In one case these piled up colonies were removed and subcultured until a cell line was derived named 13MC1 (13 Months Crocidolite 1). When these cells were injected subcutaneously in CBA/Ca mice, slow growing tumours developed which invaded the underlying leg muscle (Fig. 3.72 upper) and metastasised to the draining node (Fig. 3.72 lower).

After 3 successive passages through the subcutaneous site during which the rate of growth of the tumour increased, 13MC1 cells were injected intraperitoneally where they grew rapidly as a nodular mesothelioma-like tumour. Fig. 3.73 (upper) shows a mass of tumour nodules apparently originating in the omentum of a mouse injected with 10^6 13MC1 cells. Fig. 3.73 (lower) reveals that some of the nodules have acellular centres

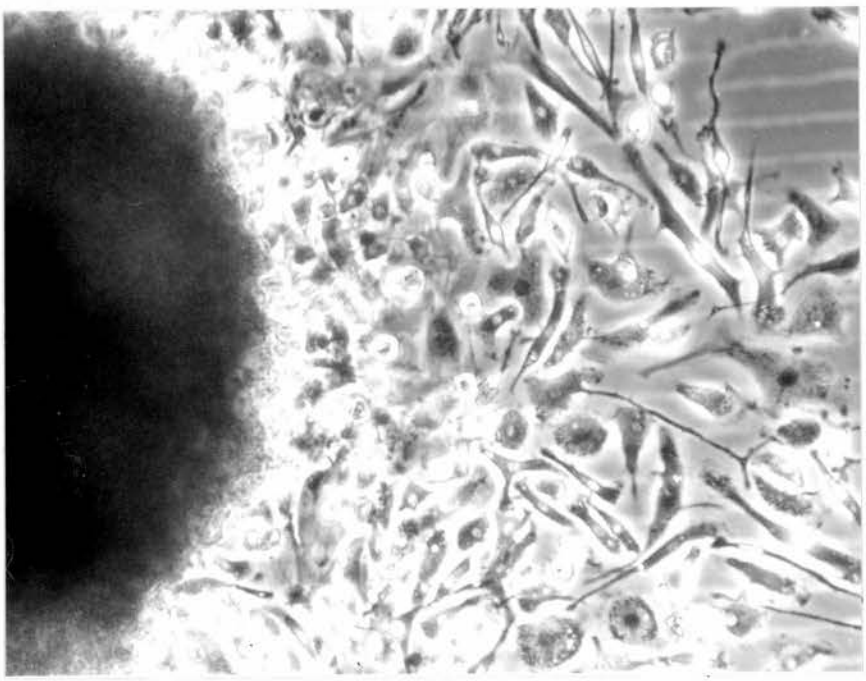


FIGURE 3.71 Piled up colony (dark area at left) with outgrowing tumour cells which grew up from ascites derived from a mouse which had been injected with crocidolite 13 months before killing. Phase contrast.

Mag. x 200

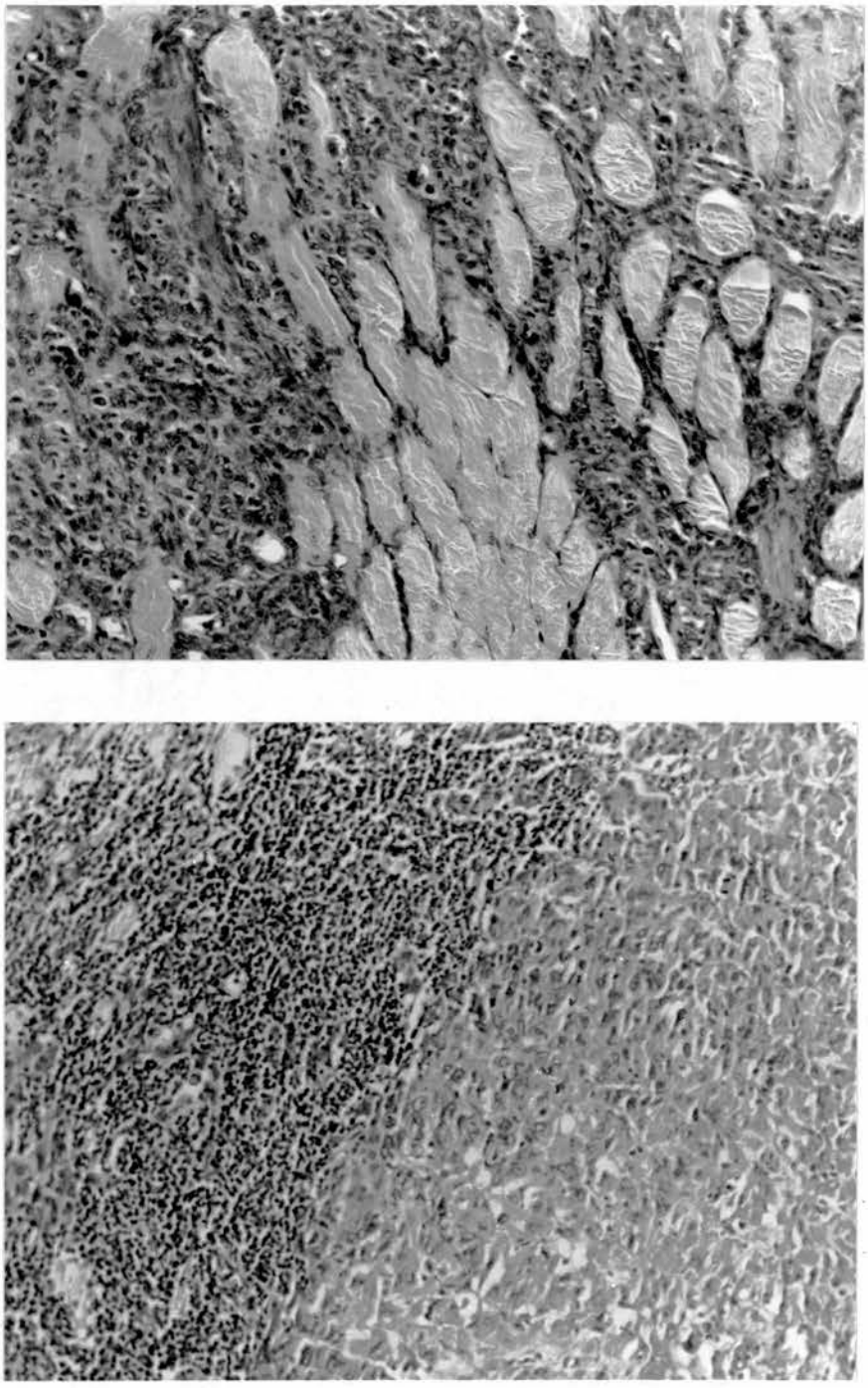


FIGURE 3.72 13MC1 tumour growing subcutaneously in a CBA/Ca mouse and showing (upper) invasion of the underlying muscle layers. Lower plate shows the lymph node draining the tumour and invasion of the lymph node by tumour cells (right) is evident.
Mag. - upper x 150; lower - x 90.

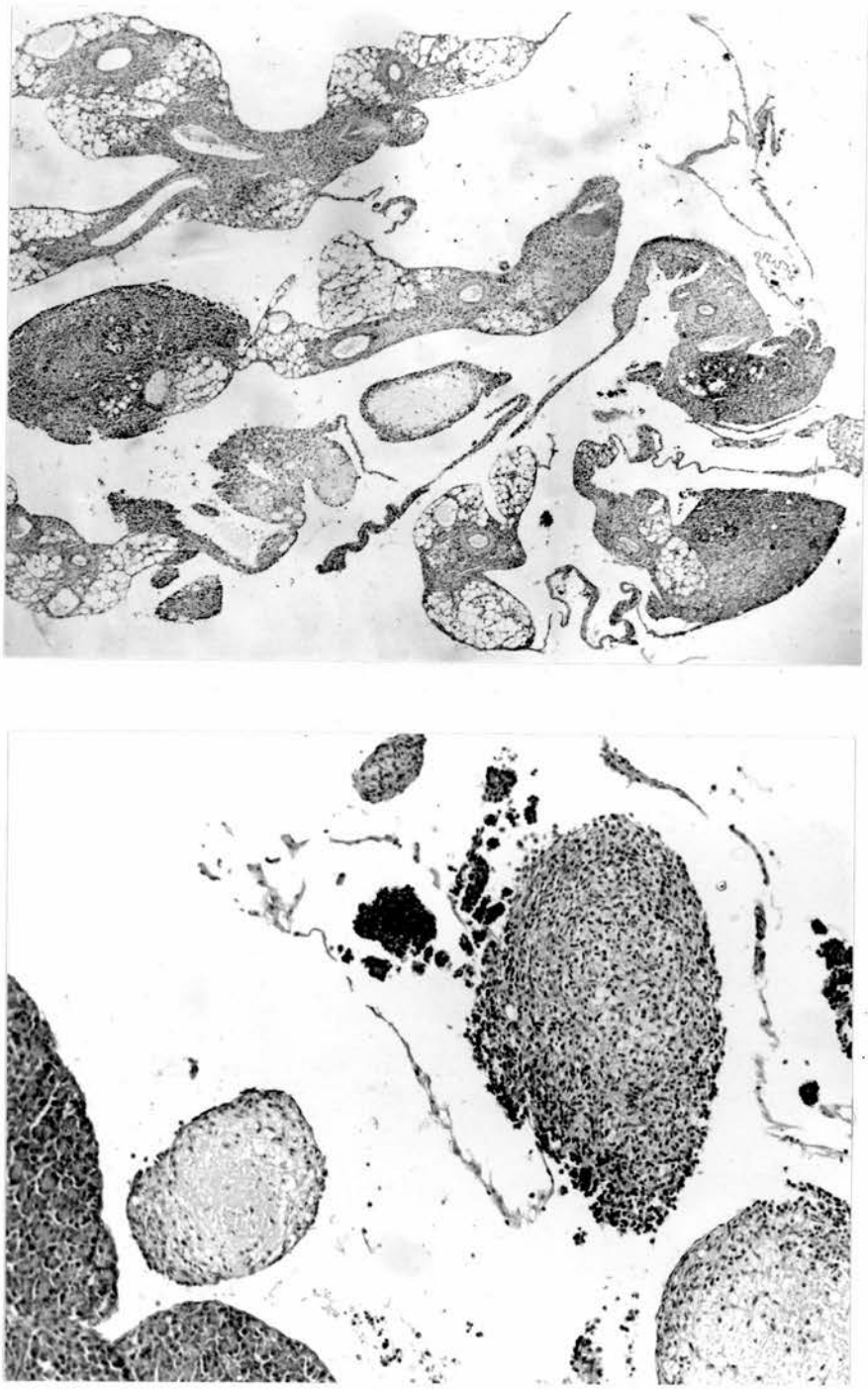


FIGURE 3.73 13MC1 cells growing intraperitoneally as a nodular tumour.
Mag. - upper x 65; lower - x 150.

while some are cellular throughout.

3.8.2.2 Ascites transfer experiments

In view of the fact that malignant cells could be grown up from the ascites developing in long term asbestos bearing mice, the direct transfer of ascites to syngeneic recipients was carried out to try and obtain an ascitic tumour which could be passaged in vivo. Table 3.30 summarises the results from these experiments where mice received 0.5 ml of ascites from animals which had developed ascites in response to ip crocidolite, chrysotile or amosite. Of 21 mice receiving ascites, 3 mice produced needle track tumours; 2 mice produced needle track tumours plus nodular tumours in the peritoneal cavity plus ascites; 1 mouse produced a needle track tumour and a peritoneal mass; 15 mice produced no pathology.

3.8.2.3 13MC1 tumour cell line

(i) Immunogenicity In order to determine whether the crocidolite induced ascites derived cell lines 13MC1, which grew in the peritoneal cavity as a mesothelioma-like tumour, was immunogenic, an immunize and challenge experiment was carried out. Fig. 3.74 shows the time to ascites development of mice unimmunised or immunised with 10^6 lethally irradiated 13MC1 cells and then challenged with 10^3-10^6 viable 13MC1 cells. In the unimmunised group there is a linear increase in the time taken to develop ascites with decreasing 13MC1 challenge. In the immunised group there were no tumour takes up to 89 days after challenge when the experiment was terminated; at autopsy, in the immunised group, there was no evidence of peritoneal tumour or ascites.

(ii) p14 membrane glycoprotein The cell membrane glycoprotein designated p14 has been claimed to be a universal marker of the transformed phenotype (Bowen and Kulatilake 1979) and an anti-serum to this component was kindly provided by Dr. D. Hannant. This anti-serum, of

TABLE 3.30 Summary of ascites transfer experiments.

EXP. NUMBER	RECIPIENTS (n)	ASCITES DEVELOPED?	TUMOUR DEVELOPED?	TIME TO DEATH OR CULL	DONOR ASCITES PRODUCED BY
1157	4	No	No	166	Amosite
1218	1	No	Needle track	143	Crocidolite
1227	4	No	No	206	Amosite
1256	2	No	No	143	Amosite
1257	2 (i)	Yes	Peritoneal nodules + needle track	75	Chrysotile
	(ii)	Yes	Peritoneal nodules + needle track	75	Chrysotile
1260	3 (i)	No	Needle track	62	Chrysotile
	(ii)	No	Needle track	91	Chrysotile
	(iii)	No	Peritoneal nodules + needle track	112	Chrysotile
1278	1	No	No	104	Chrysotile
1299	4	No	No	87	Crocidolite
TOTAL	21	2	6		

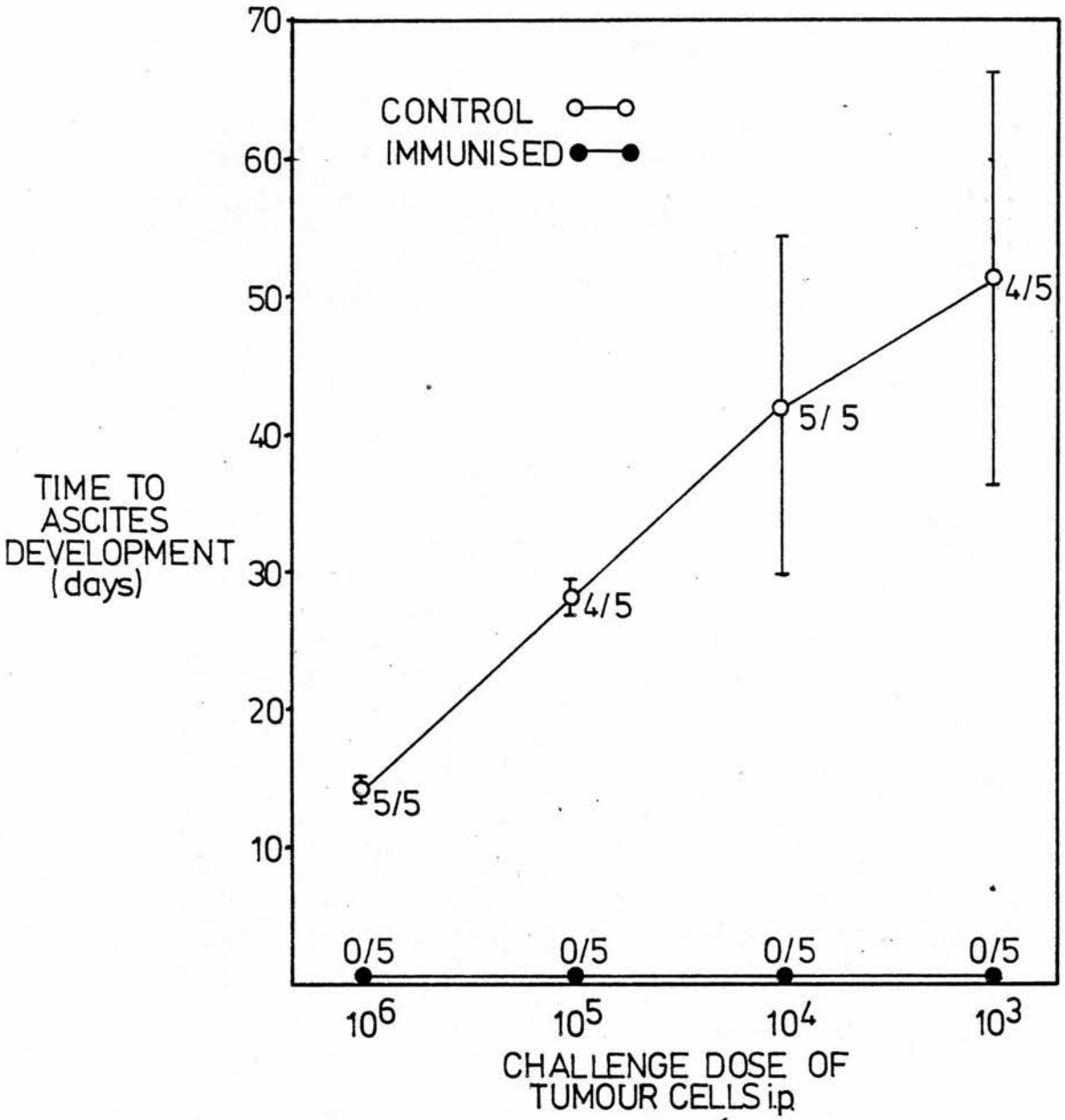


FIGURE 3.74 Effect of immunisation with 10^6 irradiated 13MC1 ascitic tumour cells on the time to ascites development following injection with 10^3-10^6 viable tumour cells. Control mice unimmunised.
Symbols denote $\bar{x} \pm$ one standard deviation.
Figures denote number of takes.

which only a small volume was available, was used in a [^{125}I] Protein A binding assay (as given in Section 2.11.5) to try and detect this component in the 13MC1 cell line. Human embryonic lung fibroblasts were used as negative controls and a range of rat mesotheliomas, derived from primary asbestos induced peritoneal mesotheliomas of various morphological type were kindly provided by Dr. R. Bolton. The data shown in Table 3.31 can only be considered to be very provisional data since it represents the results of only one experiment where 13MC1 cells were included along with the other target cells. It was not possible to repeat the experiment since supplies of the anti-serum ran out but Dr. Hannant is presently raising more anti-serum in rabbits to the pI4 component and this work will be repeated as soon as it is available. The provisional results are included because they show that binding of αpI4 to 13MC1 cells is lower than for rat mesotheliomas and the D23 hepatoma. Although the binding index of 1.0 is more than that produced by the Human Embryonic Lung cells, a binding index of 1.0 was produced by HEL cells in a separate experiment to that shown in Fig. 3.32 showing that the binding of αpI4 to 13MC1 cells falls within the range of untransformed cells.

TABLE 3.31 Binding indices of anti-pI4 serum to various targets and 13MC1 cells.

$^{+}$ Binding index derived = $\frac{\text{binding following treatment with 1/10 anti-pI4}}{\text{binding following treatment with 1/10 NRS}}$

TARGET CELLS	BINDING INDEX $^{+}$
Human Embryonic Lung Fibroblasts	0.64
Hepatoma D23	1.7
Rat Mesothelioma (a)	1.6
Rat Mesothelioma (b)	3.9
Rat Mesothelioma (c)	2.3
Rat Mesothelioma (d)	2.7
13MC1	1.0

DISCUSSION

Discussion Plan

In Chapter 4 the results of the present study will be discussed and this will be approached in two ways. In parts 4.1 to 4.3 the experiments aimed at elucidating the nature of the asbestos activated macrophage will be interpreted; these studies include measurement of activational characteristics, the use of Concanavalin A as a membrane probe and attempts to detect evidence of a neo-antigen. In parts 4.4 and 4.5 experiments into the consequences of asbestos related macrophage activation will be discussed; these experiments include interactions of asbestos activated macrophages with an experimental fibrosarcoma and the effect of asbestos activated macrophage supernatants on lymphocyte mitogenesis. Additionally in 4.6 the carcinogenic effects of asbestos on the mouse peritoneal cavity will be discussed. Finally in 4.7 the possible relevance of the present study will be discussed along with suggestions for further work.

The index numbers of the relevant sections of Chapter 3 (Results) will be given in brackets where key results are discussed.

4.1 The effects of intra-peritoneal asbestos injection on the peritoneal macrophage population

4.1.1 PEC number

In the first part of the study the effect of intraperitoneal asbestos on some membrane related characteristics of the elicited macrophage population were measured. It was apparent that the actual number of macrophages which could be harvested from the peritoneal cavity varied with time elapsed after asbestos injection. Thus there was a highly significant increase in the number of PEC at 3 days but the number at 18 days was not significantly greater than in the saline injected controls; by 70 days there was again significantly more PEC harvestable from the peritoneal cavity of asbestos injected mice (3.2.1). The majority of these cells were macrophages at each time point and at no time point was the number of PEC in the latex injected group greater than the number in the saline injected group.

The biphasic variation in the number of PEC in the asbestos injected group could represent either a real decrease in the number of cells in the peritoneal cavity at day 18 or a decrease in cells which could be lavaged by the technique used, e.g. through increased tenacity of macrophage adherence to the peritoneal viscera. The latter phenomenon has been described as the"macrophage disappearance reaction" (Shannon and Love 1980) which represents an in vivo manifestation of MIF action in delayed hypersensitivity following ip administration of an antigen to a sensitised animal. The time course of the macrophage disappearance reaction is, however, very different from the effect seen here, and peaks at 4 hours after antigen administration. Also, by definition, the macrophage disappearance reaction depends on a conventional delayed type hypersensitivity response to antigen, whereas asbestos is certainly not a conventional antigen. As discussed more fully below, in the

discussion of spreading, "MIF-like activity" can be generated by an inflammatory reaction such as is produced by asbestos; the activity in such a case is due to Bb which is the 64,000 MW fragment formed by cleavage of Factor B by bound C3b during activation of complement by the alternative pathway. Fibrin deposition on the surface of macrophages has also been implicated in the action of MIF and in the macrophage disappearance reaction (Hopper et al 1981) and the ability of asbestos to activate the clotting cascade has recently been demonstrated (Hamilton et al 1981). It is possible therefore that asbestos elicited macrophages could be immobilised by fibrin generated either directly from the asbestos activation of the clotting cascade or via secretion from asbestos activated macrophages leading to the splitting of Factor B to yield Bb. It would have to be assumed that the fibrin producing, or Bb generating activity had not peaked by Day 3 and had passed the peak by Day 70. The possibility does however exist that at both of these time points, also, some fraction of the macrophages were immobilised and therefore not harvested.

These observations focus attention on a problem inherent in studies using peritoneal cavity or alveolar macrophages, and that is the reliance on macrophages readily lavaged from the site. If different test agents produce a differential response in terms of the ability of the macrophages to adhere to the peritoneal viscera and walls, then a sampling bias is introduced. This is a bias which macrophage workers have to accept but it should be borne in mind.

4.1.2 Membrane related changes in conventionally activated and asbestos elicited macrophages

The assays chosen to study the effects of asbestos on the membrane of macrophages were chosen to reveal activational type changes and were phagocytosis, rate of spreading on glass and Fc receptor avidity; in

addition 5' nucleotidase was measured, as a check, in 3 day chrysotile induced macrophages. All of these parameters have been found to be correlated with activation and are discussed below.

4.1.2.1 Latex phagocytosis

This parameter is the least clearly correlated with macrophage activation since both decreases (Al-Ibrahim et al 1978) and increases (Nathan et al 1971) in phagocytosis have been reported following activation. It is likely that both mode of activation and surface properties of the phagocytosable particle are the major factors determining whether increased or decreased phagocytosis is expressed (David and Remold 1976). In the present study the asbestos induced macrophages had decreased phagocytic potential at day 3 following asbestos injection (3.2.2). At subsequent time points there was no difference in this parameter between asbestos, saline or latex induced macrophages despite the fact that the asbestos induced macrophages were activated as measured by other criteria. At day 3 the asbestos macrophages were most activated, as assessed by spreading, and this could have been a factor in the reduced phagocytosis; equally likely, however, is that the macrophage membrane was refractory to further phagocytosis due to recent phagocytosis of asbestos fibre. Such an effect has been demonstrated following latex (Werb and Cohn 1972) and bacterial (Pesanti and Nugent 1981) phagocytosis.

4.1.2.2 Spreading

Increased spreading on glass was present in the asbestos injected group compared to the control groups at all time points but was most marked at 3 days (3.2.4). Increased rapid spreading on glass was one of the earliest reported properties of activated macrophages (Cohn and Benson 1965) and has been described in macrophages activated in vivo with endotoxin, thioglycollate, Listeria infection and Trypanosoma

infection (Bianco et al 1976) and in vitro with muramyl dipeptide (Tanaka et al 1980), lymphokine (Adams et al 1973), alternative pathway of complement component Bb (Gotze et al 1980), the inflammatory mediator plasmin (Bianco et al 1976) and protease (Bianco et al 1980). Increased rapid spreading of rat peritoneal macrophages harvested following ip injection of crocidolite asbestos was described but not quantified by Miller (1979).

It has been suggested that the final common factor produced by this diverse range of agents which cause spreading is Bb through activation of complement via the alternative pathway (Bianco et al 1980) since purified Bb has potent spreading inducing activity. In the assay system used here with washed cells in de-complemented serum the spreading activity could be carried over from the in vivo situation as already activated membrane. It could also be stimulated in situ during the assay through the generation of plasmin as a consequence of plasminogen activator secretion as reported by Hamilton et al (1976) for asbestos activated peritoneal macrophages. Since macrophages can also secrete all the major components for activation of complement via the alternative pathway (Nathan et al 1980) and since activated macrophages also secrete C3 cleaving activity (Allison et al 1978) then local alternative pathway activation could also generate Bb.

There is a close relationship between spreading and migration inhibition and it has been suggested that MIF may act by causing direct or indirect production of Bb (Gotze et al 1980). The exact role, if any, of spreading in vivo is unclear but asbestos activated macrophages, if spread, would be unlikely to chemoattract to a further inflammatory stimulus or localised immune response; such a stasis could compromise the second response. However, rather than speculate on the possible in vivo significance of spreading it is sufficient to view in vitro spreading of macrophages as a manifestation of altered secretion of the active

components discussed above.

4.1.2.3 Fc receptor avidity

Increased macrophage Fc receptor expression, manifest as increased avidity for lightly sensitised SRBC's is a well documented index of macrophage activation (Rhodes et al 1981). Increased Fc receptor expression as measured by rosetting techniques or binding of labelled complexes has been reported in oil elicited compared to resident peritoneal macrophages (Rhodes 1975), in adjuvant activated compared to resident alveolar macrophages (Arend and Mannik 1973) and to discriminate between tumour derived macrophages, C. parvum and proteose peptone activated, and resident peritoneal macrophages (Moore and McBride 1980); rosetting techniques have also been used to detect differences in activation in foreign body granuloma macrophages (Amsden and Boros 1979). Specific rosetting techniques have also demonstrated increased avidity of Fc receptors in monocytes from tumour bearing patients compared to controls (Rhodes et al 1981) and monocytes of sarcoidosis patients compared to controls (Schmidt and Douglas 1977). Miller (1979) reported that the alveolar macrophages from rats inhaling crocidolite were activated by Fc receptor avidity. In the present study macrophage activation was present at all 3 time points as measured by the criterion of increased Fc receptor avidity (3.2.3). The activated subpopulation in the asbestos elicited macrophages were presumed to be the large cells described below (3.2.6) since, in experiments where the subpopulations of activated macrophages were separated by density gradients, the most activated cells, by Fc receptor avidity, have been the larger, denser, more rapidly sedimenting population (Walker 1974; Serio et al 1979; Moore and McBride 1980).

4.1.2.4 5' nucleotidase

As a further check on the indices of macrophage activation described above, the plasma membrane bound enzyme 5' nucleotidase was measured since the activity of this enzyme is known to be reduced to very low levels in macrophages activated by inflammatory stimuli or lymphokines (Bianco and Edelson 1978; Karnovsky and Lazdins 1978). The assay was carried out on 3 day chrysotile and saline induced macrophages. Lysosomal hydrolases, including acid phosphatase are increased on macrophage activation (Raz et al 1977) and since acid phosphatase can be assayed using essentially the same phosphate release technique, the level of acid phosphatase was also measured. The marked decrease in membrane 5' nucleotidase and increase in lysosomal acid phosphatase, in the asbestos compared to saline elicited macrophages, confirmed that the asbestos elicited macrophages were activated (3.2.5).

4.1.2.5 Morphology

Morphologically the asbestos elicited macrophages were always typified by a subpopulation of cells which were activated in appearance (3.2.6). Activated macrophages, by light microscopy are typified by their large, spread, granular and vacuolated appearance (Evans and Alexander 1976; Raz et al 1977) and the asbestos elicited macrophages, particularly at 3 days, fulfilled these criteria. The saline and latex induced macrophages did not have an activated appearance being, in general, small and unspread; when spread cells were present they did not display the granular and vacuolated appearance of the spread asbestos induced macrophages.

At scanning electron microscopy the 3 and 18 day asbestos elicited macrophages had the large, spread and ruffled appearance of cells with increased membrane activity as described by Nabarra et al (1978) in thioglycollate elicited macrophages. Increased ruffles and spreading

were described by Miller and Kagan (1976) in SEM preparations of rat peritoneal macrophages obtained following ip injection of crocidolite.

By transmission electron microscopy the 3 day asbestos elicited macrophages had an appearance very similar to 3 day thioglycollate induced macrophages with multiple vacuoles and infoldings of the surface membrane (Nabarra et al 1979). In the 70 day asbestos elicited macrophages the surface activity was reduced but there was clear morphological evidence of activation as judged by increased numbers of granules, golgi and associated vesicles, and mitochondria in keeping with the elevated secretory status of activated macrophages. The appearance of the 70 day chrysotile activated peritoneal macrophages was very similar to that described for alveolar macrophages obtained from the lungs of rats which had been inhaling crocidolite in the long term and which were found to be activated by Fc receptor and other criteria (Miller 1979).

It was noted that asbestos fibres could be detected in the vacuolar system of macrophages 70 days after initial injection. This suggested that either the macrophages which had taken up asbestos at the original challenge had a long (>70 days) residence time in the peritoneal cavity or that asbestos became available to newly recruited macrophages through death or exocytosis by previous asbestos bearing macrophages. Differing evidence currently exists as to the origin of macrophages in the mouse peritoneal cavity following an inflammatory stimulus. According to Daems and co-workers (e.g. De Baker and Daems 1981) macrophages in the peritoneal cavity mostly derive from division of pre-existing macrophages and persist in the cavity for up to 4 months. This model would certainly explain the presence of asbestos inside macrophages 70 days after injection and would suggest that a non-degradeable substance might persist in these macrophages for the remainder of the animal's lifespan. In contrast van Furth and co-workers (e.g. van Furth 1981) have evidence that the majority of peritoneal macrophages, both in the steady state and during

inflammation, originate from peripheral blood monocytes. Such a model is more dependent than the former on macrophage death or emigration from the peritoneal cavity to make room for the incoming monocytes. Although it was not quantified there was a very obvious decrease in the number of asbestos bearing macrophages between days 3 and 18 and between days 18 and 70; indeed by day 70 the presence of fibre in macrophages was a rarity in TEM sections. Thus emigration of asbestos bearing macrophages from the cavity seems to be a distinct possibility and the final destination of the dust is of particular interest. The complicating factor in such a simple consideration of macrophage kinetics in relation to dust burden was the evidence that a large amount of asbestos was sequestered in granulomas by day 70 (3.8.1). It is possible that a major part of the injected fibre was walled off in these granulomas while the remaining (? smaller fibres) asbestos could persist in the free macrophage population by division and in long term residing macrophages. This must, however, remain as speculation since macrophage kinetics in the peritoneal cavity following injection of granuloma inducing agents is unstudied.

4.1.3 The nature and extent of asbestos mediated macrophage activation

It is evident from 4.1.2 that macrophages elicited by asbestos in the mouse peritoneal cavity in both the long and short term are activated as measured by changes in several parameters; these macrophages will be henceforth referred to as asbestos activated macrophages. It is appropriate to consider what these changes reveal about asbestos activated macrophages and the place of asbestos amongst other agents known to activate macrophages.

4.1.3.1 Nomenclature

It has been said that the nomenclature of macrophage activation is "generally unclear" (van Furth 1980), in or causing "confusion" (North 1978;

Ogmundsdottir and Weir 1980) and "loosely applied" (Hopper et al 1979). This disorder in terminology has arisen due to the huge literature accumulating on the subject of macrophage activation, the multiplicity of assays used to detect activation, and the number of agents, doses and regimes used to obtain activated macrophages. There has also been a tendency to think of activated macrophages as differentiating along a well defined phenotypic line while there is good evidence that some activational characteristics can be expressed independently of others (North 1978; Nathan and Root 1977) and recent evidence tends to suggest that every activating agent produces a different activational phenotype (Werb and Chin 1981) at least expressed as the mean of the population which is the figure produced by most assays.

As a response to the obvious lack of uniformity several appeals have been made for standardisation of terminology and in general there has been a call for a 3 tier system:- 1. Resident; 2. Elicited; 3. Activated; allocation of a population to a group however has been the subject of disagreement. Karnovsky and Lazdins (1978) called for "activated" to be reserved for macrophages activated by "immunological means". The committee chaired by Morahan (1980) suggested that macrophages differing from resident macrophages in any function could enter the category of "activated"; the word activated was not apparently to be used, however, and the agent employed to produce the change should be given along with the origin and activated property. The cells used in the present study could, by this system, be "mouse peritoneal macrophages elicited 3 days after chrysotile asbestos injection for increased spreading, increased Fc receptor avidity and decreased 5' nucleotidase". By the system suggested by van Furth (1980) which has 6 categories, the same cells would be "in vivo asbestos activated macrophages as measured by increased spreading etc". Karnovsky and Lazdins (1978) and van Furth (1980) recommend that the term stimulated should be avoided but it has

frequently been used to describe activated macrophages which have not attained tumouricidal/bactericidal status (e.g. Ogmundsdottir and Weir 1980). The term elicited is to be used for procedures which accumulate macrophages and does not imply functional change although some such alteration would generally be present.

The problems in the terminology of macrophage activation are inherent in any attempt to define what is essentially a continuum of states where the place of any cell in the continuum is determined by multiple factors such as nature of stimulus, dose, state of animal, time in culture, conditions of culture etc. Seen in the light of current knowledge, what appears at first sight to be a retrograde step suggested by North (1978), may in fact be a logical one:-

> "it is desirable, therefore, to allow the meaning of the term activated macrophage to remain vague until more knowledge about the different manifestations of macrophage activation is discovered" (North 1978).

4.1.3.2 The asbestos activated macrophage as a classical inflammatory macrophage

Notwithstanding the lack of knowledge concerning the exact relationships between different indices of macrophage activation some generalisations are possible. The first is that the fully activated state is characterised by expression of tumouricidal/microbicidal function. This can be assumed to be the evolutionary "aim" of the system in mammals, i.e. to deal with invading microbes; this function has been modified, in terms of the theory of immune surveillance, to detect and eliminate tumour cells. It is also evident that resident macrophages are not optimally responsive to the lymphokine signal which triggers macrophages to attain tumouricidal/microbicidal status whereas inflammatory macrophages have undergone the functional changes which render them susceptible to lymphokine activation (Ruco and Meltzer 1978; Hibbs et al 1980). Allied to this fact may be the well documented alterations in

the response of activated macrophages to a second stimulus compared to resident macrophages. An altered response to a second stimulus, such as phagocytosis, Con A or PHA, has been reported with regard to plasminogen activator secretion (Gordon et al 1974), IL1 (Unanue 1978a), fibroblast stimulating factor (Martin et al 1981) and hydrogen peroxide release (Nathan and Root 1977). Recently a third layer of complexity has been reported with the demonstration that proteolytic enzymes prime activated macrophages to give a greater response to a triggering membrane stimulus (Johnston et al 1981). This "primed" status of activated macrophages, enabling them to react differently to resident macrophages in response to a 2^{o} or 3^{o} stimulus makes their response in vivo particularly difficult to assess due to the multiple interactions which are possible.

It is evident that asbestos is an inflammatory stimulus and so it would be expected that asbestos elicited macrophages would have the characteristics typical of inflammatory macrophages. This is borne out by the activational parameters measured which show asbestos activated macrophages to be similar to macrophages induced by sterile irritants such as thioglycollate or oil which also have increased spreading, decreased 5' nucleotidase (Cohn 1978) and increased Fc receptor avidity (Rhodes 1975). The differentiation signals involved in asbestos mediated macrophage activation can only be guessed at but direct activation of macrophages in de-complemented serum has been reported for asbestos (Davies et al 1974) and such direct activation has recently been attributed to release of endogenous C3b by macrophages (Schorlemmer et al 1981) which then acts as an autostimulator for macrophage activation; alternatively asbestos has been shown to activate the complement (e.g. Saint Remy and Cole 1980) and kinin (Hamilton et al 1981) cascades which are also capable of generating activating intermediates.

As harvested, classical inflammatory macrophages are not tumouricical (Hibbs et al 1978; Cohn 1978) and the use of an in vitro tumour cell

killing assay showed that asbestos activated macrophages were not tumouricidal to any degree which could be considered to have any relevance in vivo (3.3.1.1). The asbestos activated macrophage can therefore be considered to be a classical inflammatory macrophage.

It is necessary here to consider another problem of nomenclature in macrophage activation concerning the tumouricidal status of activated macrophages which has relevance to the nature of asbestos activated macrophages. The apparent conflict arises from (i) the frequent assertion that inflammatory macrophages from the peritoneal cavity are not, or only slightly, tumouricidal (Hibbs et al 1978; Meltzer et al 1979; Hopper et al 1979) and (ii) reports, principally by Schorlemmer and co-workers, that macrophages can be activated by inflammatory generating agents which activate complement by the alternative pathway to generate C3a which has potent cytolytic activity for tumour cells (Ferluga et al 1978; Schorlemmer et al 1977a; Allison et al 1978) and is less active against normal cells (Temple and Allison 1980). This apparent conflict can be resolved by making the distinction between (i) chronic and (ii) transient, low grade inflammatory producers. Schorlemmer and co-workers deal with chronic inflammation inducers such as zymosan, streptococcal cell walls and LPS, all of which activate complement by the alternative pathway and which have many effects on inflammation associated cells; in terms of eliciting inflammatory macrophages into the peritoneal cavity, however, the usual agents are thioglycollate, proteose peptone, oil and caseinate which do not activate complement and produce only mild and transient inflammation. This is another case of loose terminology in relation to macrophage activation. Since asbestos does activate complement by the alternative pathway (Wilson et al 1977; Hasselbacher 1979; Saint Remy and Cole 1980) it would be predicted that asbestos activated macrophages would generate C3a and therefore possess tumouricidal function but they do not.

In this connection it is of note that tumouricidal activation has been described for subcutaneous glass coverslip elicited macrophages (Poste 1979). Glass has many properties similar to asbestos, being a silicate mineral, non-degradable, carcinogenic (Brand et al 1976) and having the capability to activate the alternative pathway via plasmin through activation of the clotting system (Ryan and Majno 1977). Poste reported that between days 4 and 7 after implantation of coverslips there was up to 44% macrophage cytotoxicity towards a tumour line in vitro.

One possible explanation for the failure of asbestos activated macrophages to express tumouricidal activity could lie in the reported ability of prostaglandin E to inhibit tumouricidal function (Schultz et al 1978). It is clear that the level of prostaglandin E secreted by activated macrophages is sufficient to cause self-inhibition of tumouricidal function under appropriate conditions (Taffet and Russell 1980). In keeping with the sequential triggering concept in activated macrophages, which was discussed above, Bonney et al (1981) have shown that Prostaglandin E release by activated macrophages can be increased by latex phagocytosis for up to 4 days after removal from the animal. Asbestos has been reported to cause prostaglandin release by macrophages (Humes et al 1977; Sirois et al 1980) and it is possible that persistent activation of macrophages by asbestos both directly and via the complement system, allied to the huge phagocytic stimulus produced by asbestos (1.6×10^9 fibres/mg in UICC crocidolite - Brown et al 1978) could result in prolonged prostaglandin secretion and thereby inhibition of tumouricidal function. A second possible mechanism whereby asbestos could inhibit tumouricidal function is through the agency of trace elements including cadmium, which have recently been demonstrated to inhibit macrophage mediated tumour cell cytotoxicity (Nelson et al 1982). Cadmium is one of the many trace elements present in the UICC asbestos samples and these

elements, leaching from fibre within macrophages, could inhibit tumouricidal function. A third explanation for the lack of tumouricidal activity relies on the lymphokine theory of macrophage activation to the tumouricidal state. If, in vivo, lymphokine is mandatory for attainment of the tumouricidal state with inflammatory macrophages as the target population required, then the vital lymphokine step is missing in the model used here. The effect of asbestos treatment on the lymphokine secretory status of lymphocytes in vivo is unknown but Miller et al (1979) have reported alveolar macrophage activation and manifestations of cellular immunity in the lungs of rats inhaling crocidolite asbestos; lymphokine levels were not, however, measured. This cellular immunity showed some properties of antigen specific T cell proliferation by sensitised lymphocytes in response to a putative membrane neo-antigen on asbestos dusted macrophages. As discussed later there was no evidence in the present study of a humoral response to a putative neo-antigen in asbestos activated peritoneal macrophages while attempts to detect the neo-antigen using xenogeneic, heterologous anti-sera failed due to low specific anti-macrophage activity. If increased lymphokine is generated in the asbestos inhalation model either by the above mentioned antigen specific T. cell proliferation, or due to a generalised adjuvant effect, this does not seem to be the case in the peritoneal cavity following asbestos injection since the tumouricidal function was not present. This could be due either to the use of the peritoneal site or the single injection model compared to the multiple exposures produced by a daily inhalation regimen.

Depressed levels of lymphokine production would be predicted from the suppressor effects of asbestos activated macrophage supernatants discussed below (4.5).

Scanning electron microscope studies of tumour cell/macrophage interactions revealed that non-tumouricidal saline and asbestos elicited

macrophages did not spread out on tumour cells but remained rounded with a small area of membrane contact; C. parvum macrophages, which were tumouricidal, could be seen to be spread out on dead and dying tumour cells (3.3.1.3). Such close interaction between tumour cells and tumouricidal macrophages has been reported in other transmission electron microscope studies (Hanna et al 1976) and scanning electron microscope studies (Kaplan and Seljelid 1977) and it is possible that such a close interaction is necessary for tumour cell killing.

4.1.3.3 Existing evidence that asbestos can activate macrophages

The current state of knowledge with regard to the effects of asbestos on macrophages other than cytotoxic effects, is shown in Table 4.1 and reveals that if the in vitro dose is low enough or the macrophages are derived from animals treated with asbestos in vivo by inhalation or injection then there is abundant evidence of macrophage activation. At the start of this study, in 1979, only selective release of lysosomal hydrolases (Davies et al 1974) and plasminogen activator release (Hamilton et al 1976) were reported as markers of activation following in vitro or in vivo treatment of peritoneal macrophages with asbestos; only the work of Miller (Miller and Kagan 1976; Miller et al 1979) suggested that alveolar macrophages could become activated following asbestos inhalation. The present study has confirmed the macrophage activating potential of asbestos using different criteria and has shown that, at least in the model used, the macrophages were not tumouricidal. In addition, during the course of the study, further evidence for the macrophage activating potential of asbestos has been published (Table 4.1).

4.2 The use of Concanavalin A as a probe in macrophage/asbestos interactions

Lectins have been used extensively as probes of membrane structure and function in biological research. Due to their specificity for

TABLE 4.1 Published evidence that asbestos can activate macrophages

MACROPHAGE SOURCE	ASBESTOS		INDEX OF ACTIVATION	REFERENCE
	DOSE	TREATMENT		
Mouse Peritoneal Proteose Peptone Induced	Chrysotile $2.5\text{-}15\ \mu g/10^6$	In vitro	Selective release of lysosomal enzymes	Davies et al 1974
Mouse Peritoneal Asbestos Elicited	Chrysotile 10-100 µg ip	In vivo	Plasminogen activator release	Hamilton et al 1976
Mouse Peritoneal Endotoxin Elicited	Chrysotile $5\text{-}50\ \mu g/10^6$	In vitro	Plasminogen activator release	Hamilton et al 1976
Rat Peritoneal Asbestos Elicited	Crocidolite 50 mg ip	In vivo	Size increase Spreading on glass	Miller and Kagan 1976
Rat Alveolar	Crocidolite 1350 fibres/ml	In vivo	Size/morphology Spreading Fc receptor avidity Stimulation of lymphocytes to take up thymidine	Miller et al 1977-81
Rat Alveolar	Chrysotile 100 µg/ml	In vitro	Release of fibroblast stimulating factor	Bitterman et al 1981
Guinea Pig Alveolar	Chrysotile $100\ \mu g/10^6$ cells	In vitro	Prostaglandin release	Sirois et al 1980
Mouse Peritoneal Asbestos Elicited	Crocidolite Chrysotile 2.5 mg ip	In vivo	Spreading Fc receptor avidity 5' nucleotidase activity Size/morphology	Present Study

carbohydrate groups and their large size lectins do not traverse the membrane except by endocytosis; their association with cells under controlled conditions to prevent endocytosis is therefore directly proportional to the amount of specific carbohydrate moieties at the cell surface. While titration of surface carbohydrate groups is one use of lectins, the cross-linking of specific binding sites at the cell surface by the valencies of the lectin is the property utilised in the other major application of lectins, i.e. as membrane perturbing agents. Lectins, on binding and cross-linking receptors, can engender transmembrane signals which elicit a sequence of metabolic events which, depending on the cell in question, culminate in for example transformation in lymphocytes, or stimulation of oxygen metabolism in activated macrophages.

Concanavalin A is a lectin derived from Canavalia ensiformis and is composed of 4 identical sub-units each comprising 237 amino acids and one binding site for the specific carbohydrate α D mannose/α D glucose (Cunningham et al 1976). This means that Con A is tetravalent with regard to its carbohydrate binding sites and can cross-link adjacent groups either within the cell surface milieu or between adjacent cells causing them to agglutinate. Con A in particular and also other lectins have been used extensively to show differences between cells in terms of their surface glycoproteins; for example between transformed and untransformed cells (review by Noonan 1978; Nicolson 1976b). Macrophage plasma membranes have been investigated using a number of lectins and have been reported to bind many of them including Con A (e.g. Oliver and Berlin 1976), Lotus tetragonolobus lectin (Hamma et al 1981), wheat germ agglutinin (Hadley et al 1977), phytohaemagglutinin and pokeweed mitogen (Loor and Roelants 1974). In addition macrophage differentiation has been shown to be associated with differences in the expression of cell

surface carbohydrates which can be detected by lectins, e.g. differences in the amount of wheat germ agglutinin binding between monocytes and mature macrophages (De Water et al 1981) and expression of a novel ∝ glactose-rich glycoprotein receptor, which binds Griffonia simplicifolia I-B4 isolectin, in activated but not resident macrophages (Maddox et al 1982).

Con A has been particularly utilised to investigate its binding, or receptor, glycoprotein in the macrophage membrane and has been used in studies on the effects of spreading and phagocytosis on Con A binding sites (Lutton 1973) and the effect of the pollutants ozone and nitrogen dioxide on alveolar macrophage Con A binding sites (Goldstein et al 1977). The macrophage Con A receptor glycoprotein has itself been analysed and found to be a transmembrane glycoprotein (Adachi et al 1980) as would be expected from its ability to be mobilised by the cytoskeleton when it interacts with these elements at the inner face of the membrane. These cytoskeletal associations of the Con A receptor glycoprotein have received attention in studies on the ability of the receptor to "cap" under suitable conditions (i.e. incubation with FITC-Con A at 37°C) as has been demonstrated in other cells such as neutrophils and lymphocytes (Oliver and Berlin 1976). Thus 30% of resident alveolar macrophages (Williams et al 1977), 0% of resident peritoneal macrophages (Pick and Wilner 1979) and approximately 10% of caseinate elicited peritoneal macrophages (Williams et al 1979) have been reported to cap on incubation with FITC-Con A. The role of the cytoskeleton has been confirmed by the general increase in caps found following colchicine pre-treatment (Oliver and Berlin 1976; Williams et al 1977; Pick and Wilner 1979; Williams et al 1979). With microfilament disrupting agents there is a more complex response with increases in the proportion of capping alveolar macrophages (Williams et al 1977; Williams et al 1979) but no ability to increase caps in peritoneal macrophages (Williams et al 1979; Pick and Wilner 1979).

In the present study Con A was utilised in three ways:-

(i) to visualise the specific membrane glycoprotein (Con A receptor glycoprotein) following interactions between asbestos and macrophages;

(ii) to study the capping of Con A receptors in asbestos activated macrophages;

(iii) to enumerate the Con A receptors on macrophages using $[^{125}I]$ Con A.

4.2.1 Effect of asbestos interaction with the macrophage membrane on the Con A receptor glycoprotein

This study was conceived to test the frequent assertion, made principally by Harington and co-workers (Harington et al 1975; Harington 1976) that asbestos may interact with cell membrane glycoproteins causing clustering and leading to osmotic disturbance. This is seen as the underlying mechanism in haemolysis, "early macrophage cytotoxicity" in serum free conditions and in phagolysosomal rupture in late macrophage cytotoxicity in the presence of serum (Harington 1976). Using light microscopy and FITC labelled Con A to visualise the Con A binding sites there was occasional evidence that the Con A receptor glycoprotein was concentrated or clustered at areas of contact between fibre and macrophage membranes. This was manifest as areas of bright fluorescence coinciding with areas of contact between fibres and macrophages (3.4.1). These accumulations of fluorescence were, however, rare events despite the many cells obviously undergoing interactions with fibre. This suggested that there was something special about these interactions which did show evidence of clustering. The most likely candidate in this matter was size, with only large fibres being resolved by bright field and large fibres causing the greatest membrane clustering; these two factors could combine resulting in association of clustering with large fibres. There were, however, many more cases where large fibres were interacting with membrane in absence of any change in the pattern of

fluorescence. It seems possible also that the disturbance could be artefactual representing the trapping of FITC-Con A between fibres and membrane or selective adsorption of FITC-Con A on to fibre in contact with membrane. Cells showing clustering of fluorescence at points of contact with fibre were so rare, within a population where so many cells were in contact with fibre in the absence of clustering, that artefact was a distinct possibility. The possibility of artefact is further increased by the presence of clusters in both serum free and serum containing conditions when an effect could only be expected in the serum free conditions since the active Mg groups are rapidly protein coated and masked in protein solution.

Transmission electron microscopic preparation of macrophage/ chrysotile interactions, with the Con A binding sites demonstrated ultrahistochemically, also failed to reveal convincing evidence of accumulation of Con A binding sites at points of membrane/fibre contact (3.4.1.2). This result is similar to that found for another inert particulate, latex, where no alteration in the pattern of cell surface Con A binding sites was observed at points of attachment between particle and membrane (Eguchi et al 1979). It appears that, with respect to the macrophage membrane Con A receptor glycoproteins, there is no effect on their disposition on attachment to the membrane of a non-toxic particulate (latex) or a toxic one (chrysotile) although it remains possible that the reaction was too coarse to reveal any very fine effects.

4.2.2 Con A induced capping in asbestos activated macrophages

4.2.2.1 Role of activation in Con A induced capping

Experiments to determine the proportion of macrophages which formed caps on incubation with FITC-Con A revealed that both C. parvum and asbestos activated macrophages had significantly increased numbers of cells which capped on incubation with FITC-Con A (3.4.2.2). This figure was

32% for C. parvum and chrysotile activated macrophages and 22% for crocidolite activated macrophages; control macrophages had 4% capping cells. Since latex elicited macrophages had control levels of capping it is clear that phagocytosis alone is not always sufficient stimulus for inducing the capping sub-population. Since proteose peptone elicited macrophages also had low levels of spontaneously capping cells, then not all macrophage activating agents induce an increased capping population.

The finding of significantly increased levels of capping macrophages in resident peritoneal macrophages from nude mice compared to saline induced macrophages of ordinary mice (3.4.2.9) confirmed that capping is related to activation since nude mice have macrophages which have been reported to be in a constant state of activation (Zinkernagel and Blanden 1975). However, the testing of an early adhering population, putatively the most activated macrophages, did not demonstrate a higher percentage of capping cells (3.4.2.6).

Evidence that macrophage activation plays a role in Con A capping exists in previously published work:- thus Pick and Wilner (1979) reported that resting peritoneal macrophages had 0% capping cells while Williams et al (1979) using peritoneal macrophages induced with caseinate, a procedure likely to cause some macrophage activation, found approximately 10% capping macrophages. Similarly the finding of 30% capping guinea pig alveolar macrophages is in keeping with the suggestion that resident alveolar macrophages are in a consistently higher state of activation than resting peritoneal macrophages (Hopper et al 1979).

4.2.2.2 Role of the cytoskeleton in Con A capping

Pharmacological modification of capping was carried out using the microfilament disrupting drug cytochalasin B and the microtubule disrupting drug colchicine. In the present study colchicine had virtually no effect in promoting capping (3.4.2.7) although previous studies have demonstrated

that, with colchicine treatment the level of Con A capping can be increased 2-30 fold (Williams et al 1977; Williams et al 1979; Pick and Wilner 1979). It is clear however that in all of these studies the majority of macrophages were not colchicine sensitive being either spontaneously capping or non-capping despite colchicine treatment. In the present assay system, with CBA mouse peritoneal macrophages, the mobility of the Con A receptor glycoprotein is clearly not inhibited by colchicine sensitive microtubules. It seems likely that, as suggested by Pick and Wilner (1979), there are sub-types of microtubules and microfilaments which are not colchicine or cytochalasin B sensitive and the proportion of these elements may be liable to species and sub-species variation. The abolition of capping in activated macrophages following cytochalasin B treatment (3.4.2.7) confirms that microfilaments are necessary for capping in peritoneal macrophages (Williams et al 1979) and spleen cells, presumed to be lymphocytes (De Petris 1974).

The finding that lignocaine inhibited capping in activated macrophages (3.4.2.7) is in keeping with the previously reported cytoskeletal disrupting (Nicolson et al 1976) and capping inhibitory (Ryan et al 1974) properties of tertiary amine anaesthetics such as lignocaine.

Experiments demonstrating that Con A receptors are regenerated after 4 hours with both saline and chrysotile elicited macrophages revealed that there was no fundamental defect in the membrane receptor generative ability of chrysotile activated macrophages compared to control macrophages (3.4.2.8). This timing is also in agreement with Lutton (1973) who reported regeneration of Con A receptors $4\frac{1}{2}$ hours after phagocytosis of latex by macrophages as measured by recovery of $\left[^{125}I\right]$ Con A binding.

4.2.2.3 Electron microscopy of capped macrophages

At scanning electron microscopy large bleb-like protrusions were present on the surface of a proportion of chrysotile induced macrophages

(3.4.2.10) which had been treated with Con A under capping conditions; these blebs were absent from similarly treated saline induced macrophages. The secondary electron emission from these blebs was different from the rest of the cell suggesting that the physicochemical make up of that portion of membrane was different from the rest of the cell; such a difference could be due to the accumulation of Con A receptor glycoproteins in the "cap".

Saline induced macrophages treated with Con A under capping conditions showed, at scanning electron microscopy, an increase in macrophages with leafy, frond-like processes in keeping with an increase in membrane activity.

By transmission electron microscopy the already complex membrane infoldings of 3 day chrysotile activated macrophages was seen to be increased, on a qualitative basis, when these cells were treated with Con A under capping conditions (3.4.2.10). Also the number of cells showing extreme membrane infoldings was approximately doubled. It thus appears that asbestos in vivo has a "Con A-like" effect on the macrophage membrane, which is emphasised by treatment with Con A in vitro. As discussed previously there are probably many factors generated by acute asbestos inflammation in the peritoneum which could have some of the membrane perturbing properties of Con A (e.g. plasmin, other proteases, Bb) and which could prime the macrophage membrane to further perturbation by Con A. By transmission electron microscopy no structures analogous to caps were seen in relevant populations although caps have been described at transmission electron microscopy in alveolar macrophages treated with Con A (Williams et al 1977). It seems likely therefore that not enough sections were examined to obtain a good section of a cap since these would be relatively rare (3.4.2.10).

4.2.2.4 The possible role of Con A recptor number and position in capping

It was possible that the transmembrane signal engendered by Con A

binding, and which lead to capping, could be switched on in activated macrophages due to an increased number of receptor sites on such cells. The relative number of Con A binding sites on the surface of control and activated macrophages was therefore assessed by binding of $[^{125}I]$ Con A. In initial experiments the finding of a significant reduction in $[^{125}I]$ Con A binding following latex phagocytosis was in agreement with the original experiments of Lutton (1973); this suggested that our assay system which was based on Lutton's was comparable.

The total $[^{125}I]$ Con A binding to asbestos activated macrophages was the same as for control macrophages (3.4.3.4). Previous work concerning the effect of environmental pollutants on lectin binding to alveolar macrophages revealed no effect of ozone or nitrogen dioxide on binding of tritiated thymidine Con A compared to controls (Goldstein et al 1977); Hadley et al (1977) however found increased binding of the lectin wheat germ agglutinin to ozone treated macrophages. C. parvum activated macrophages bound approximately 30% more $[^{125}I]$ Con A than control and asbestos activated macrophages (3.4.3.4). Since activation can occur via several different pathways and the interaction of the various differentiation signals can be very complex (as discussed in 4.1.3.2) it is not surprising that there is considerable variation in activational phenotypes (Hopper et al 1979; Werb and Chin 1981). Due to the very different natures of asbestos and C. parvum it is likely that they activate macrophages by different pathways. As discussed above, asbestos and C. parvum activated macrophages do possess different activational phenotypes with C. parvum macrophages being tumouricidal while asbestos activated macrophages are not. The increased binding of $[^{125}I]$ Con A to C. parvum macrophages could therefore reflect functionally related differences in the membrane of tumouricidally activated macrophages. Increased binding could also be due to increased cell surface poly-

saccharide as reported for macrophages involved in cell-mediated immunity (Hammond and Dvorak 1972). Notwithstanding these explanations for increased numbers of Con A binding sites in C. parvum activated macrophages it was apparent that Con A receptor number was not the major factor in capping since asbestos activated macrophages, which capped, bound the same amount of $[^{125}I]$ Con A as control macrophages, which did not have a large capping subpopulation.

It was possible that the differences in capping potential of control and activated macrophages might reside in the exact position of the Con A receptor glycoprotein. Although the Con A receptor glycoprotein has been shown to be predominantly transmembrane in organisation (Adachi et al 1980) it was possible that in control macrophages there might be an increased proportion of peripheral membrane, or even glycocalix situated, Con A binding glycoproteins, while in capping macrophages the transmembrane organised Con A binding glycoprotein, able to link with the cytoskeleton, might predominate. Differences in the protease releasability of Con A bound to surfaces of control and transformed fibroblasts have been reported suggesting that proteases can detect fine qualitative differences in the Con A receptor sites within the complex milieu of the cell surface. Using trypsin on control and activated macrophages, however, revealed no difference in the bound $[^{125}I]$ Con A which was trypsin releasable (3.4.3.5).

An alternative explanation for the mechanism of capping in activated macrophages lies in the increased oxidative metabolism frequently reported in such macrophages (review Nathan 1982). Oliver et al (1977) demonstrate that H_2O_2, the important microbicidal and tumouricidal agent, could have a direct role in tubulin oxidation in polymorphonuclear leukocytes by causing inhibition of microtubule assembly and thereby promoting capping. Nathan and Root (1977) have reported that activated macrophages respond to membrane perturbing agents, such as Con A or phagocytosis, by

release of H_2O_2. Thus the capping in activated macrophages could be due to increased H_2O_2 production in these cells. This explanation of capping in activated macrophages is an attractive one despite the failure in the present study to show a role for colchicine sensitive structures in capping and the reported differences in cytoskeletal requirements for capping between macrophages and polymorphonuclears (Williams et al 1979).

4.2.2.5 Phagocytosis of different particulates and $[^{125}I]$ Con A binding

Three different particles were used to study the effect of phagocytosis on $[^{125}I]$ Con A binding:- latex, opsonized SRBC's and asbestos. It was hoped to compare the percent membrane internalization, as measured by decrease in $[^{125}I]$ Con A binding, on phagocytosis of these different particles. This was done in order to test the hypothesis that macrophages phagocytosing fibres might exceed the normal constraints on the proportion of membrane which can be internalized and might start to internalize transport areas of membrane which are normally selectively excluded from phagosomal membrane (Tsan and Berlin 1971). This did not prove possible due to the persistent finding of increased binding of $[^{125}I]$ Con A following asbestos phagocytosis (3.4.3.3) presumed due to binding of $[^{125}I]$ Con A to surface adherent and partly phagocytosed fibre. The study did however reveal that ingestion of opsonized red cells did cause reduction in $[^{125}I]$ Con A binding (3.4.3.2). It was of interest, however, that treatment of macrophages with red cell lytic buffer alone caused marked reduction in binding of $[^{125}I]$ Con A. This has some relevance for studies of membrane related parameters following removal of contaminating red cells with lytic buffer since a recovery period in culture may be necessary to allow regeneration of the glycoproteins stripped from the cell surface by treatment with the buffer.

4.3 Investigations on a putative neo-antigen expressed in the membrane of asbestos activated macrophages

Miller and co-workers (Miller and Kagan 1977; Miller et al 1979; Miller and Kagan 1981) have provided indirect evidence that asbestos inhalation, in the long term, can cause alveolar macrophage activation and expression of an asbestos related neo-antigen. This neo-antigen is then envisaged as being recognised by the host immune system as foreign so precipitating a cell mediated immune response with sensitised T lymphocytes being present in the spleen of dusted animals; co-culture of spleen cells and macrophages from dusted rats is then analogous to an antigen specific T cell proliferation and thymidine is incorporated (Miller and Kagan 1981). Wahl et al (1980b) have shown that fibroblast stimulating factors are produced during antigen specific T cell proliferation and so the above events could be important in asbestos related lung fibrosis. The indirect evidence that the lymphocyte proliferation is antigen specific resides in the demonstration of macrophage/T cell rosette formation (Miller et al 1979) which is similar to that produced during conventional antigen specific T cell/macrophage interactions (Lipsky and Rosenthal 1975) and the demonstration that the proliferation is not due to aldehyde moieties on the macrophage surface (Miller and Kagan 1981). There was a non-specific lymphoproliferative component in these reactions and the role of diffusible factors was not investigated, the data being interpreted on the basis of membrane changes only.

In the present study it was appropriate therefore to try and detect evidence of neo-antigen expression in asbestos activated peritoneal macrophages and this was attempted in two ways:-

(i) By raising xenogeneic anti-sera to control and asbestos activated macrophages and attempting to demonstrate differences in specificity

following suitable absorption;

(ii) By attempting to detect increased surface Ig in asbestos activated macrophages as evidence of an auto-immune humoral response to the putative neo-antigen; indirect evidence for such a humoral response was obtained by Miller and Kagan (1977) as increased immune adherence in alveolar macrophages from rats which had inhaled crocidolite.

4.3.1 Use of anti-sera raised against asbestos and saline induced macrophages

Anti-sera contained significant activity against CBA/Ca mouse thymocyte (3.6.1) and this activity could be absorbed out with thymocytes. Activity against PEC remained (3.6.2) but was again reduced when only adherent PEC (macrophages) were used as targets (3.6.3). When the anti-macrophage activity was found to be present experiments were carried out to ascertain whether the binding assay, routinely carried out in preliminary experiments at 1/50 dilution of anti-sera, represented saturation binding conditions. Saturation binding was required for comparison of the anti-sera specificities but even at 1/10 dilution saturation binding was not present with the standard number of target macrophages used (3.6.4). Less target cells were tried but the counts became so low in the tests that they were not significantly greater than background binding given by the pre-bleeds. The low level of specific anti-macrophage activity caused the experiment to be abandoned.

Immunofluorescence failed to demonstrate any qualitative differences in the two types of anti-serum with regard to pattern of binding to the two macrophage types (3.6.5). Complement mediated lysis also revealed a low level of specific anti-macrophage activity in the anti-sera (3.6.6).

No adequate explanation can be offered for the low level of anti-macrophage activity present in the rabbit anti-sera. There was abundant evidence of immune response to common mouse antigens as shown by the high

level of binding of the anti-sera to thymocyte and which could be absorbed out with thymocytes. Similarly there was a high level of binding to PEC but this was greatly decreased when only adherents were used as targets; it appears therefore that the greatest part of the activity was directed against non-macrophage components of the PEC, i.e. lymphocytes and polymorphs. Essentially two methods of raising xenogeneic anti-macrophage sera have been used with either cells emulsified in Freunds adjuvant (Unanue 1968; Isa 1974) or whole cells (Panijel and Cayeux 1968; Hirsch et al 1969; Kaplan and Mohanakumar 1977) as the immunogens; both immunization regimes yielded specific anti-sera. If the present study can be repeated both immunization schedules will be used and also more rabbits, in the hope of obtaining specific anti-macrophage sera of high activity. Ideally monoclonal antibodies to control and asbestos activated macrophages would be obtained to try and detect differences (see 4.7.2).

4.3.2 Attempts to detect surface Ig on asbestos activated macrophages (3.7)

In an attempt to detect a humoral response to the putative neo-antigen an anti-mouse Ig serum was used in combination with [^{125}I] Protein A. 6, 14 and 294 day crocidolite and saline elicited macrophages were used and no difference was ever detected between crocidolite and saline macrophages with regard to surface Ig (3.7). By the criterion of surface Ig therefore there was no evidence of a neo-antigen.

4.3.3 Macrophage activation and membrane antigen modulation

It is useful to review the published data on membrane antigenicity in activated macrophages with specific reference to the concept of an asbestos related macrophage neo-antigen and ask two questions:-

(i) is there any evidence that macrophage activation by other agents causes alteration in membrane antigenicity?

(ii) is there any reason to suspect an immune response to such neo-antigens?

In answer to the first question there are several studies which have revealed altered antigenicity in activated macrophages compared to resident macrophages. Kaplan and Mohanakumar (1977) reported that an anti-serum to P388 D1 cells (activated macrophages by many criteria) was cytotoxic to pyran and C. parvum activated macrophages but not to glycogen or thioglycollate activated or resident macrophages. Waldrep et al (1981) using relevant anti-sera demonstrated new antigens in a lymphokine treated macrophage cell line that was absent from untreated cells suggesting that a macrophage differentiation signal was capable of inducing expression of a new antigen(s). Using direct biochemical methods, differences in membrane glycoproteins have also been demonstrated between resident and activated macrophages. Yin et al (1980) using lacto-peroxidase/glucose oxidase labelling demonstrated qualitative and quantitative differences in the membrane glycoproteins between resident, thioglycollate and endotoxin activated macrophages. A similar result was reported by Kaplan and Olstad (1981) using biosynthetic labelling of membrane determinants, with both qualitative and quantitative differences evident between resident and ascites activated macrophages. Pearlstein et al (1978), using lactoperoxidase iodination of resident and starch elicited macrophages detected a novel 195,000 MW membrane glycoprotein expressed by the starch elicited macrophages, but absent in the resident macrophages. Indirect methods, using lectins, have also yielded evidence for differences in membrane glycoprotein between monocytes and macrophages (De Water et al 1981) and between resident and activated macrophages (Maddox et al 1982).

It is evident therefore that conventional activating agents cause altered antigenicity or increases in existing antigens, the latter being

difficult to disprove, and so the expression of neo-antigen on asbestos activated macrophages might be expected. It remains possible, however, that alveolar macrophages might be less liable to antigenic modulation than peritoneal macrophages, which have been discussed so far, since Davies and Stossel (1981) found only small differences between resident and adjuvant elicited alveolar macrophages with regard to membrane proteins; they did however observe that their technique did not have high resolution.

If new antigens are expressed by activated macrophages in response to endogenous differentiation signals in vivo the question then arises as to whether such a finely evolved host defensive response would evoke an autoimmune response. There is no reference in the PAGE/SDS studies, discussed above, of large amounts of 50K or 25K components which could represent heavy or light chain respectively; it is true however that the time span for eliciting activated macrophages may not be sufficiently long for a full autoimmune humoral response to develop. It has recently been suggested that autoimmunity is a far more common occurrence than was previously supposed with, for instance, around 50% of B lymphocytes, following immune response to a conventional antigen, secreting IgM specific for IgG (rheumatoid factor) (Dresser 1978). Similarly a common type of immunostimulation, malarial infection, has been shown to result in high levels of autoimmunity including antibodies against the membrane of parasitized red cells with generation of specific T helper and B cells (Rosenberg 1978). The adaptive value of such autoimmune responses could lie in regulation of immune response (idiotype networks) and in opsonization for rapid clearance, e.g. in the case of parasitized red cells.

In view of the above facts it seems likely that asbestos activated macrophages, and other kinds of macrophages activated in vivo in the course of host defence, could elicit autoimmune responses; the absence

of evidence for such a response in the present study could be explained on the basis of the peritoneal site or the single exposure model used.

If other diseases in the lung, e.g. pneumonia, which result in both activated macrophages with neo-antigens and autoimmunity, do not have fibrosis as a sequel than it is necessary to look for another factor associated with asbestosis. It seems likely that a major factor is the persistence of the dust and further work is required to assess whether the fibroblast stimulation in asbestosis is due to a factor (i) released by asbestos activated macrophages or (ii) lymphocyte derived due to antigen specific T cell proliferation to neo-antigens on the former; both could have a role.

In parts 4.4 and 4.5 experiments will be discussed which examined the possible consequences of asbestos induced macrophage activation for host defence. These experiments essentially involved tumour growth in vivo and in vitro following asbestos treatment and associated experiments (4.4) and the effects of asbestos activated macrophage supernatants on lymphocyte mitogenesis and associated experiments (4.5).

4.4 Effects of asbestos on an experimental fibrosarcoma model

As part of the study into the activational status of asbestos elicited macrophages the in vitro tumouricidal activity was measured. It should be stressed, however, that interactions of asbestos activated macrophages with tumour cells is of fundamental importance in terms of the carcinogenic effects of asbestos and so a series of experiments were carried out on this subject. These experiments were aimed at measuring the effects of asbestos activated macrophages and asbestos on tumour growth in vivo and in vitro.

As discussed previously (4.1.3.2) the asbestos elicited macrophages were not apparently cytotoxic to tumour cells in a tumour cell killing assay. In addition the supernatants from 3 day asbestos activated

macrophages were tested in an *in vitro* assay to measure cytostatic effects on tumour cell proliferation. Such cytostatic factors have been reported to be released from activated macrophages (Keller 1981) and would not be detected by a pre-labelling killing assay but would be detected by a post-labelling proliferation assay. There was however no evidence for the presence of cytostatic factors in the supernatants of asbestos activated macrophages, nor from saline, latex or *C. parvum* elicited control macrophages (3.3.2). Since *C. parvum* activated macrophages were always tumouricidal in the tumour cell killing assay, the cytostatic assay therefore revealed that direct interaction between *C. parvum* macrophages and targets was required and that a diffusible cytotoxic factor, similar to that described by Sharma *et al* (1980), was not responsible for *C. parvum* macrophage killing of tumour cells.

As might be expected from the *in vitro* cytocidal and cytostatic assays, the in *vivo* experiments where asbestos was injected into mice and the growth of a concomitant tumour monitored, failed to show any consistent effect of asbestos on tumour growth. An intriguing finding however was that 5 mg chrysotile ip caused small significant reductions in tumour growth while 2.5 mg and 20 mg did not (3.3.3.1). Using the same regimen, *C. parvum* injection caused significant reduction in tumour growth but altering the regimen to one which profoundly increased the ability of *C. parvum* to inhibit tumour growth caused 5 mg chrysotile to lose its effect. Both 5 and 20 mg of crocidolite ip failed to cause reduction in tumour growth. These results are difficult to interpret but one interpretation is that the mechanism whereby 5 mg chrysotile caused reduced tumour growth was different from the mechanism whereby *C. parvum* inhibited tumour growth. Numerous studies have shown that C. parvum acts in large part through the generation of tumouricidal macrophages and so it follows that 5 mg chrysotile must operate through some other mechanism possibly through a general adjuvant effect on other anti-tumour responses

e.g. NK cells.

The Winn assay experiments tended to confirm the general trend present in other assays, that ip asbestos did not generate tumouricidal macrophages. Control and chrysotile induced PEC mixed with tumour cells at 10:1 and 100:1 then injected subcutaneously revealed no significant differences in tumour growth (3.3.4). This suggested that the difference between site of primary macrophage activation (peritoneal cavity) and tumour growth (subcutaneously on leg) was not a major impediment to the manifestation of any tumouricidal/tumouristatic effects in the in vivo system described above. As a further check however, crocidolite was mixed with the tumour cell inoculum so that local macrophage activation at the site of the tumour could further promote macrophage/tumour cell interactions. Crocidolite was chosen because of its lower surface activity than chrysotile so reducing the chances of direct toxic effects of the fibre on the tumour cells and in addition it was coated by incubation with FCS. These experiments yielded a direct dose related decrease in tumour size with increasing amounts of crocidolite in the inoculum (3.3.5). While it was possible that local activation of tumouricidal or tumouristatic macrophages had occurred at the site of the tumour due to crocidolite, it remained possible that the crocidolite had exerted a direct dose dependent toxicity on the tumour cells in the inoculum; the effect of crocidolite and crocidolite leachate was therefore tested on tumour cells in culture. Both crocidolite and chrysotile and leachates were found to have very little effect on tumour cells in culture except at the highest doses of both crocidolite and crocidolite leachate, which were markedly inhibitory (3.3.6). This high dose of crocidolite (100 µg/well) was similar to, or less than the equivalent dose of crocidolite which the tumour cells in the inocula were exposed to. This raised the distinct possibility that the inhibition of tumour growth produced by including asbestos in the

inocula was indeed a direct toxic effect of asbestos on the tumour cells and local crocidolite mediated activation of macrophages to a tumouricidal or tumouristatic state does not have to be invoked.

The effect of asbestos and asbestos activated macrophages in the fibrosarcoma model used here can be summarised as:-

(i) using in vitro assays asbestos activated macrophages were not appreciably tumouricidal nor did they release factors which were tumour cell cytostatic

(ii) in Winn assays chrysotile induced PEC were not inhibitory or stimulatory to tumour growth

(iii) ip injection of crocidolite never caused significant effects, compared to controls, on concomitant tumour growth while the single intermediate dose of chrysotile (5 mg) which caused inhibitions of tumour growth did not appear to bring about its effect via macrophages.

(iv) although crocidolite mixed with tumour cells and inoculated subcutaneously caused inhibition of tumour growth this could be explained by a direct toxic effect of crocidolite on the tumour cells which was evident at similar doses in in vitro experiments.

It can be assumed therefore that asbestos in CBA/Ca mice, neither inhibits nor potentiates the growth of an experimental fibrosarcoma through the agency of macrophages.

The ability of asbestos to activate macrophages to the tumouricidal state may be intuitively unexpected since asbestos is a carcinogen. However glass can cause macrophage activation to the tumouricidal state (Poste 1979) and is tumourigenic (Brand et al 1976). Schistosome eggs have also been found to be tumourigenic (Brand et al 1976) while macrophages from schistosome egg granulomas have been reported to be activated (Amsden and Boros 1979) and although the tumouricidal function was not assayed for, it is very likely that they are tumouricidal. Thus it would not be unprecedented for asbestos to cause tumouricidal macrophage

activation and also, eventually, tumours. The relationship between inflammation and tumour growth is not clear and asbestos is both a chronic inflammation generating agent and a carcinogen. Data is conflicting with Fauve et al(1977) reporting that a non-specific inflammatory stimulus, magnesium silicate in calcium phosphate, in the feet of mice, caused increased resistance to challenge with Lewis lung carcinoma cells. Nelson et al (1981) and Nelson and Nelson (1981) used a variety of mitogens as inflammatory inducing agents and reported that while some of these agents were able to suppress tumour growth, their ability was not clearly related to apparent intensity of inflammatiom and some of the agents enhanced tumour growth. It has been suggested that inflammatory mediators may have an important role in tumour growth and that macrophage secretions such as plasminogen activator and proteases may be central (Vassalli et al 1977). Many, if not all promoting agents are powerful inflammatory generating agents and there may be a parallel between the ability of some producers of chronic inflammation to eventually cause fibroblast division and fibrosis, and the ability of promoters to cause tumour cells to divide; macrophages could be important cells in this model as discussed in (1.12.2). Asbestos has many of the properties of a promoter (e.g. Topping and Nettesheim 1980) and asbestos activated macrophages release plasminogen activator (Hamilton et al 1976) and proteases, which have been implicated in maintenance of the transformed phenotype (Quigley 1979). The possibility that macrophages might contribute to the actual transforming event was suggested by the work of Weitzman and Stossel (1981) who showed that phagocytes (neutrophils and monocytes) were mutagenic in Ames type bacterial mutation tests. The authors suggested that oxygen metabolites, H_2O_2 and O_2^-, were responsible, and activated macrophages are a particularly rich source of these metabolites (Nathan 1982). The accumulation of macrophages so characteristic of asbestos inflammation could create

ideal conditions for the elevated levels of such substances.

Clearly further work is necessary to elucidate the relative importance, the in vivo significance and the factors controlling which role a given macrophage population plays in tumour growth, i.e. (i) tumour cell killer; (ii) releaser of tumour growth promoting factors; (iii) releaser of tumourigenic oxygen metabolites. The activational state is clearly a likely major factor in which of these phenotypes, if any, is dominant and the role of asbestos is of particular interest.

4.5 Suppressor action of asbestos activated macrophage supernatants on lymphocyte mitogenesis

In view of the central role of the macrophage in immunoregulation and the alteration of function on activation (reviewed in 1.11.1) it was appropriate to consider the possible altered role of the asbestos activated macrophage in immune responses.

The best characterized and most reported of a family of lymphocyte modulating factors released by macrophages (Diamenstein et al 1979) is IL1 first described by Gery and Waksman (1972) in mice although it has also been described in humans (Diamenstein et al 1979). IL1 has frequently been demonstrated through its ability to potentiate Con A or PHA mitogenesis in thymocytes (Gery and Waksman 1972; Blyden and Handschumaker 1977; Beller and Unanue 1980) and it can also replace macrophages in helping antibody synthesis by B cells (Koopman et al 1978). IL1 has been extensively characterised and purified (Blyden and Handschumaker 1977; Mizel 1979; Economou and Shin 1980).

In the original description of macrophages as the source of IL1 Gery and Waksman (1972) noted that agents which stimulated macrophages increased IL1 production. Subsequently activated macrophages have been used as a source of IL1:- alveolar macrophages, BCG elicited, LPS treated in vitro

(Murphy et al 1980), P388 D1 cells treated with phorbol myristate acetate (Mizel 1979), peritoneal macrophages resident or thioglycollate elicited treated with LPS in vitro (Tenu et al 1980), human peripheral blood monocytes treated with LPS in vitro (Blyden and Handschumaker 1977). It seemed relevant therefore to examine the asbestos activated macrophage supernatants for IL1 activity.

In order to calibrate and optimise the thymocyte assay several different conventional methods of obtaining IL1-rich macrophage supernatants were tried including LPS treatment in vitro for 24 and 48 hours and induction of activated macrophages in vivo with proteose peptone and Con A; no IL1 activity was ever detected in these supernatants (3.5.1). In an attempt to discover the reason for this failure to detect IL1 activity in these supernatants a search of the literature was undertaken and a large number of publications was discovered demonstrating a suppressor factor present in supernatants from activated macrophages (reviews by Nelson 1976; Schechter et al 1980). Reviewing the data from the above experiments, aimed at detecting IL1 activity, revealed abundant evidence of suppression of mitogenesis; it was apparent therefore that the conditions under which supernatants were generated, or the conditions of the thymocyte mitogenesis assay, were such that suppressive activity was being detected in activated macrophage supernatants.

Using saline and latex elicited macrophages as controls, and asbestos elicited macrophages, supernatants were prepared and tested in the sub-optimal Con A mitogenic assay. Doses of 1 and 2 μg/ml were found to best detect suppression and so these were used routinely.

Both crocidolite and chrysotile induced macrophage supernatants were found to be significantly inhibitory to thymocyte mitogenesis when the data were expressed as mitogenic indices at 1 and 2 μg/ml Con A; chrysotile was significantly inhibitory to mitogenesis at 2 μg/ml Con A (3.5.4). The inhibition produced by 50% asbestos supernatant in the

incubation mixture was approaching 50% in some assays suggesting that almost complete abolition of mitogenesis could be produced by carrying out the assay in 100% supernatant. Neither latex nor saline elicited macrophages released suppressor activity into supernatants (3.5.4). The inhibition of mitogenesis produced by asbestos supernatants was dose related (3.5.5). The inhibitory effect of chrysotile supernatant was an acute effect with macrophages obtained 280 days after chrysotile injection showing no suppressor activity in their supernatants and indeed with there being a significant stimulatory effect present; However, 280 day saline injected macrophages also showed a stimulatory effect and so the former effect was age specific rather than asbestos specific (3.5.6).

In order to test whether the suppressive agent was actively secreted by asbestos activated macrophages or whether it was passively released by dead or damaged macrophages, control macrophages were treated with a lethal dose of chrysotile in vitro. The centrifuged supernatant was then tested in a mitogenesis assay and found not to be inhibitory to thymocyte mitogenesis (3.5.6) and so it could be assumed that the suppressor factor(s) was a secretion from macrophages and not a cell component released due to toxic effects on the macrophages. In an attempt to characterise the suppressor factor(s) a sample of chrysotile activated macrophage supernatant was halved and one half dialysed overnight before testing along with the undialysed aliquot in the thymocyte mitogenesis assay. Dialysis was found to abolish suppression at 1 μg/ml Con A and reduce suppression by more than half at 2 μg/ml Con A (3.5.8). This suggested that the major suppressive component was < 16,000 MW but that there was also a minor, large, non-dialysable, suppressor component.

Experiments on the effect of the asbestos macrophage supernatants on tumour cells (3.3.2) and fibroblasts (3.5.9) showed that the supernatants

were not inhibitory to these cells so confirming that the inhibitory effect was lymphocyte specific. Further evidence in support of lymphocyte specificity was demonstrated by the finding of significant suppressor effects of the supernatant on mature T lymphocytes (nylon wool column purified splenocytes) (3.5.10). The suppressor factor was not detectable in the serum of mice 3 days after asbestos injection (3.5.11) over and above the suppressor activity which is expected in control serum and which is well documented (Nelson 1976). It was possible that asbestos exocytosed by asbestos activated macrophages during preparation of the supernatant and too small to be spun out by centrifugation, could be exerting a direct suppressive effect on the thymocyte mitogenesis assay; the effects of asbestos on thymocyte mitogenesis were therefore tested. While both crocidolite and chrysotile were inhibitory to background proliferation neither was inhibitory to mitogenesis at 2 and 4 µg/ml Con A (3.5.12). Chrysotile, however, was significantly stimulatory to mitogenesis at 2 and 4 µg/ml Con A. Thus asbestos in the asbestos activated macrophage supernatants could not be responsible for the suppression. The fact that chrysotile could cause stimulation of mitogenesis could have some significance in vivo and requires further study.

This part of the study has revealed that asbestos activated peritoneal macrophages release a factor into the supernatant which is inhibitory to lymphocyte mitogenesis. Such factors have been previously reported to be released by peritoneal macrophages activated in vivo with such diverse activating agents as peptone (Keller 1976), C. parvum (Wing and Remington 1977), T. gondii infection (Wing and Remington 1980) and LPS (Nelson 1976). In addition activated macrophages have been shown to inhibit a number of proliferation independent lymphocyte functions such as MIF release (Varesio and Holden 1980), MAF release (Varesio et al 1981) and cytotoxic T cell generation (Klimpel and Henney 1978) suggesting that inhibition of mitogenesis is indicative of a more relevant

and generalised suppression which may have relevance in vivo.

Unlike lymphocyte activating factor, the macrophage derived suppressor factor(s) have not been well characterised. Nelson et al (1976) reported that activated macrophages from endotoxin treated mice produced a suppressor factor which was removed by dialysis and was replaced by potentiation of mitogenesis. Essentially the same result was found by Calderon and Unanue (1975) using supernatants from peptone induced macrophages, who reported the suppressor factor to be $<14{,}000$ MW (Calderon et al 1974). In the case of the asbestos macrophage derived inhibitory activity, the active principle was largely dialysable but removal of the inhibitory component did not reveal potentiating activity.

The events which lead to an activated macrophage expressing either inhibitory or potentiating activity to lymphocyte proliferation have been the subject of discussion (Nelson 1976; Nelson et al 1976) and it is likely that since both enhancing and inhibitory factors may be present in a supernatant then the sum of these two may determine the functional activity of the supernatant. In addition, ratio of macrophages to lymphocytes has been shown to be a major factor in co-culture experiments (Wing and Remington 1977); the exact role of such a variable in the case of supernatants is complex. Time in culture has been shown to have a profound effect on the ability of activated macrophages to be suppressive (Wing and Remington 1980) when during the first 24 hours of culture inhibitory activity was manifest, while during subsequent culture stimulating activity was present. The functional state and nature of the target lymphocytes is also of obvious importance and Wadee and Rabson (1981) have demonstrated that suppressor supernatants from activated macrophages operate by switching on suppressor T cells in the target population; obviously the origin of the lymphocyte population,

with regard to the presence of such cells, is of central importance in whether a suppressor supernatant can operate via this pathway.

A role for prostaglandin, albeit not fully understood, has been suggested by Schechter et al (1980) and a prostaglandin producing suppressor cell has been described (Goodwin et al 1977). In addition Mullink and von Blumberg (1980) have shown that drugs which inhibit prostaglandin biosynthesis are inhibitory to macrophage dependent suppression of PHA lymphocyte mitogenesis. It seems likely that some of the inhibitory effects of activated macrophages could therefore be explained on the basis of prostaglandin release by these cells. Furthermore some of the conflicting evidence of inhibition by activated macrophages could be due to the reported effects of different tissue culture buffers on prostaglandin release by macrophages (Brune 1980). The reported ability of asbestos to activate peritoneal macrophages to release prostaglandin (Humes et al 1977; Sirois et al 1980) makes it possible that these ubiquitous agents could be involved in suppression of lymphocyte mitogenesis by asbestos activated macrophages.

Suppression of cell mediated immunity has been frequently reported in asbestotics (Kang et al 1974; Kagan et al 1977(a); Haslam et al 1978; Wagner et al 1979; Gaumer et al 1981) and a macrophage derived factor could have a role in these findings. In the present study no evidence was found for excess inhibitory activity in the serum of 3 day asbestos injected mice although Kagan et al (1977a) described an inhibitor of PHA mitogenesis in the serum of some asbestotics.

4.6 The carcinogenic response of the CBA/Ca mouse peritoneal cavity to asbestos injection

The experiments relating to development of ascites and production of tumour cell lines from asbestos injected mice were outwith the mainstream of the present study and so discussion of the experiments and results

will be brief.

Classical, solid, multinodular mesotheliomas were never produced by asbestos injection in the CBA/Ca mouse model in the present study although such tumours have been produced in mice in previous studies (Davis 1974b; Wirth 1975). Ascites did, however, develop and was rapidly fatal; ascites bearing animals were therefore killed as soon as abdominal distension was detected. The time taken to ascites development was not obviously dependent on the asbestos type or dose, with all ascites developing within 400-600 days after injection (3.8.2.1). Transfer of ascites to normal syngeneic hosts did not reliably result in tumour or ascites development (3.8.2.2) although solid tumours, needle track tumours and ascites did develop occasionally. The ability of these occasional ascites to grow as tumours was not obviously related to the asbestos type which caused the original ascites (3.8.2.2). When ascites was grown in culture to provide a permanent cell line, 13MC1, this was found to be tumourigenic with fibrosarcomatous morphology in the subcutaneous site and to be mesothelioma-like with nodules and ascites when injected intraperitoneally (3.8.2.1). In the peritoneal cavity 13MC1 grew in a well defined manner with time to development of ascites being inversely proportional to number of tumour cells inoculated ip. Immunising with lethally irradiated 13MC1 cells provided resistance to subsequent challenge with tumour cells demonstrating that the 13 MC1 cell line was immunogenic (3.2.2.3). Provisional data from $[^{125}I]$ Protein A binding assay revealed 13MC1 cells to be low or negative with regard to expression of the surface marker designated p14 and reported to be a universal marker of malignancy (Bowen and Kulatilake 1979) (3.2.2.3).

Mesothelioma cell lines have been previously derived from rat asbestos induced tumours and grown in nude mice and in culture to study their morphology (Gormley et al 1980b). The characteristics of rat

asbestos induced mesotheliomas have also been studied by passage through syngeneic rats and by growth in culture (Brown et al 1980). These workers reported that neither groups immunised following surgical excision of primary tumour nor by implantation of irradiated tumour fragments, showed resistance to challenge with viable tumour cells. Further evidence for lack of immunogenicity in mesotheliomas was provided by Embleton et al(1976) who failed to detect lymphotoxicity to allogeneic mesothelioma cells in the peripheral blood of mesothelioma patients. Cole et al (1982) however, reported immune responses to rat asbestos induced peritoneal mesotheliomas manifest as specific lymphotoxicity in peripheral blood cells to allogeneic mesothelioma cells. In the present study the asbestos induced mesothelioma-like cell line 13MC1 was immunogenic with 10^6 immunising irradiated cells holding back 10^6 challenging viable tumour cells.

Immunogenic tumour specific transplantation antigens have been described as a major feature of chemically induced tumours (Baldwin 1976) and virally induced tumours (Moore 1978). The present study therefore does not preclude either chemical-type carcinogenesis in asbestos carcinogenesis, as suggested by Craighead and Mossman (1982), or a viral aetiology, as suggested by Kanazawa et al (1979) for mesothelioma. As a test of the possible ability of asbestos to cause expression of tumour viruses, CCH_1 tumour cells were incubated in the presence of crocidolite then injected subcutaneously; a control group were injected with the same number of tumour cells which had not been incubated with crocidolite. Any effect of asbestos in causing increased tumour virus expression would be expected to be evident as increased growth of the tumour cells when injected subcutaneously but there was no difference in rate of tumour growth between the crocidolite incubated and control groups (3.3.5).

It seems possible that the conflicting findings of immunogenicity of mesotheliomas could be due to differences in site of origin of mesothelioma since Brown et al (1980) used pleural derived experimental mesotheliomas and found them non-immunogenic while both Cole et al (1982) and the present study used peritoneal experimental mesotheliomas and found evidence of immunogenicity. Provisional data on the presence of the postulated common marker of malignancy, designated pI4, in 13MC1 cells suggested that it was present in lower quantities than in either rat hepatomas or rat mesothelioma cell lines. The pI4 component has been suggested to be present in all tumour cells (Bramwell and Harris 1978; Bowen and Kulatilake 1979) and low expression or absence from 13MC1 cells would be of great interest. However the data must be regarded as provisional until more anti-pI4 can be obtained and extensively tested in the $[^{125}I]$ Protein A binding assay against 13MC1 cells.

4.7 Conclusion

4.7.1 Relevance of the present study to asbestos related disease

The present study was undertaken to investigate the effects of asbestos on macrophages in vitro with special reference to the macrophage membrane. If such a study is to be of any relevance the results should shed some light on the possible events occurring when asbestos exposure leads to disease in humans and so the mouse peritoneal macrophage model must reasonably meet the following criteria:-

(i) the rodent is a valid model for extrapolation to humans;

(ii) the macrophage is a valid cell to study in relation to the aetiology of asbestos related disease;

(iii) the peritoneal macrophage is a valid alternative to the alveolar macrophage for such studies.

Condition (i) has been amply documented to be satisfied since

asbestos inhalation by rodents results in development of interstitial fibrosis, lung carcinoma and mesothelioma (reviewed in 1.8). Condition (ii) is satisfied in view of the importance of the macrophage in control of fibroblast proliferation, immune response and tumour growth (reviewed in 1.11). Condition (iii) does present particular difficulties since it has been frequently shown that alveolar macrophages do behave differently from peritoneal macrophages in important respects (reviewed in 1.11.1.4). Notwithstanding these differences, the demonstration in the present and previous studies that both a carcinogenic and fibrogenic response can be elicited in the peritoneal cavity with asbestos, suggests that, in important respects, the peritoneal macrophage functions in ways similar to the alveolar macrophage.

If the extrapolation is justified, therefore, the major findings of the present study indicate that:-

In general asbestos inhalation could result in macrophage activation in the lung with all the corolleries for altered secretion and function which activation carries with it for inflammation, fibroblast proliferation, immunoregulation and tumour development (reviewed in Chapter 1).
In particular:-

(i) the asbestos activated macrophage may not inhibit or potentiate, directly, the growth of tumour cells (but see ii);

(ii) the asbestos activated macrophage may release an immunosuppressive factor (possibly prostaglandin E) causing local or generalised immunosuppression; this macrophage derived factor could have a role in the suppression of cell mediated immunity which has been consistently documented in asbestosis (reviewed in 1.6.1); immunosuppression could be a factor either directly or indirectly in tumour development and immunoregulation of fibroblast proliferation.

(iii) the ability of asbestos activated macrophages to spontaneously cap with Con A suggests that altered membrane function may have

important consequences for other membrane related functions not investigated here, e.g. antigen presentation; capping may also be indicative of increased H_2O_2 and O_2^- release (as discussed in (4.2.2.4) which could cause epithelial damage and prolong inflammation.

4.7.2 Suggestions for future research

During the preparation of the present study many suggestions for further work have arisen with regard to the role of the macrophage in asbestos related disease. Some general comments will therefore be made with regard to potential future direction of research in this field.

1) Although a peritoneal macrophage model was used in the present study alveolar macrophages must be viewed as the cells of choice to study the effects of asbestos on macrophages; however peritoneal macrophages are convenient alternatives and much valuable and relevant information can be obtained through the intraperitoneal injection model. Utilising this model, multiple injections could be used to more realistically mimic the sequential exposure produced by daily inhalation and a number of secondary variables could be introduced such as relevant activating agents (e.g. bacteria known to infect the lung), surfactant, animal age etc. Asbestos inhalation experiments should be carried out, although they are much more expensive than intraperitoneal exposure, and various aspects of structure and function in the elicited macrophage population should be studied.

2) The mechanism(s) whereby asbestos activates macrophages is of obvious importance. Several pathways exist for activation including (i) auto-stimulation through C3b, proteases etc; (ii) externally mediated, i.e. through direct action of asbestos in activating complement by the alternative pathway so generating C3b. This would be of particular importance if clinical control of progressive disease was to be considered.

3) While many aspects of macrophage secretion in relation to activation are worthy of study it is relevant to pick out a few which are of particular interest. Oxygen metabolites, H_2O_2 and O_2^-, are released by activated macrophages and these could have an important role in epithelial damage and persistence of inflammation. Secretion of H_2O_2 is also liable to the activation/triggering model whereby activated macrophages release increased amounts of any secretion but treatment with a triggering agent can increase this release many fold. The questions which can be asked are:-

(i) can asbestos activated macrophages release increased H_2O_2?

(ii) can they be triggered by conventional membrane perturbing agents to increase release?

(iii) can asbestos act as a triggering agent to conventionally activated macrophages?

The same questions can be posed for release of fibroblast stimulating activity which is also liable to sequential triggering and has particular relevance to asbestos fibrosis.

4) The presence of neo-antigens in asbestos activated macrophages was not elucidated in the present study although there was no evidence for an obvious humoral immune response to these cells. In order to determine whether the fibroblast proliferation so typical of asbestosis is due to release of fibroblast stimulating factors from activated macrophages or from antigen specific T cell proliferation in response to neo-antigens on the former, or both, is a major target for study. It should be possible to add membrane preparations or even separated membrane fractions to lymphocytes from dusted animals and detect antigen specific T cell proliferation; it would be relevant to try and detect fibroblast stimulating activity in the supernatants. More indirectly, conventional anti-sera and monoclonal antibodies could be raised to membrane preparations from asbestos activated macrophages whose specificity and ability to

block antigen specific T cell proliferation to asbestos related macrophage neo-antigens could be demonstrated.

5) Although the results of this present study were essentially negative with regard to effects of asbestos activated macrophages on tumour growth this type of study could be extended. In particular the interaction of such macrophages with more relevant tumour models, e.g. carcinoma cell lines, mesothelioma cell lines. Again, as in all of these studies, alveolar (or pleural) macrophage/tumour cell interaction would be of particular relevance.

In view of some evidence suggesting that tumour promotion can inhibit activation to the tumouricidal state it would be of particular interest to ascertain whether asbestos activated macrophages can attain the tumouricidal state on treatment with appropriate differentiation signals, e.g. lymphokine, LPS.

6) With regard to direct effects of asbestos on membrane glycoproteins the present study did not reveal convincing evidence that direct interactions between chrysotile asbestos and the macrophage Con A receptor glycoprotein caused clustering of the latter. Ideally this effect would be studied by freeze fracture which reveals the disposition of membrane glycoproteins with high resolution and great clarity. Those experiments carried out to try and assess whether fibre phagocytosis compromised the normally well conserved topographic distribution of macrophage membrane function were a failure. These postulated effects would be better studied by measuring specific transport, e.g. adenisine or lysine during and following phagocytosis of asbestos and a conventional particulate.

7) Further work should be carried out to characterise the immunosuppressive factor released by asbestos activated macrophages.

8) In addition to the foregoing it is necessary to examine the full range of immune functions which macrophages perform with regard to

their expression in asbestos activated macrophages, e.g. antigen presentation, helper function in lymphocyte proliferation, quantitative Fc and C3 receptor mediated immune phagocytosis.

These studies would help clarify the role of the asbestos activated macrophage in asbestos related diseases.

REFERENCES

ACHESON, E.D. and GARDNER, M.J. (1979a) The ill effects of Asbestos on Health. in Asbestos, Vol. 2 Final report of the advisory committee: papers commissioned by the committee Health and Safety Commission HMSO 7-83.

ACHESON, E.D. and GARDNER, M.J. (1979b) Mesothelioma and exposure to mixtures of chrysotile and amphibole asbestos. Arch. Env. Health 34 240-242.

ADACHI, I., KATAGIRI, T., TERAO, T. and OSAWA, T. (1980) Concanavalin A receptor proteins of plasma membranes of guinea pig macrophages Cell Struct. Funct. 5 247-257

ADAMS, D.O., BIESECKER, J.L. and KOSS, L.G. (1973) The activation of mononuclear phagocytes in vitro: immunologically mediated enhancement. J. Reticuloendothel. Soc. 14 550-570

ADAMS, D.O. and SNYDERMAN, R. (1970) Do macrophages destroy nascent tumours? J. Nat. Cancer Inst. 62 1341-1345

ADAMS, D.O., MARINO, P.A. and MELTZER, M.S. (1981) Macrophages from C3H/Hej and A/j mice have different deficits in expression of tumoricidal function. in Forster and Landy Eds. 1981, 414-417

AL IBRAHIM, M.S., VALENTINE, F.T. and LAWRENCE, H.S. (1978) Activated lymphocytes depress phagocytosis of latex particles by human monocyte macrophages. Cell Immunol. 41 217-230

ALLEN, D.H. and DOE, W.F. (1981) Studies of pulmonary alveolar macrophage function in diffuse interstitial lung disease (DIL.D) Aust.N.Z. J. Med. 11 102-103

ALLISON, A.C. (1971) Lysosomes and the toxicity of particulate pollutants. Arch. Int. Med. 128 131-139

ALLISON, A.C. (1973) Experimental methods - cell and tissue culture effects of asbestos particles on macrophages, mesothelial cells and fibroblasts. in Bogovski et al Eds. 1973, 89-93

ALLISON, A.C. (1978) Mechanisms by which activated macrophages inhibit lymphocytes. Immunol. Rev. 40 3-27

ALLISON, A.C., CLARK, I.A. and DAVIES, P. (1977) Cellular interactions in fibrogenesis. Ann. rheum. Dis. 36 (Suppl.) 8-11

ALLISON, A.C., FERLUGA, J., PRYDZ, H. and SCHORLEMMER, H.U. (1978) The role of macrophage activation in chronic inflammation. Agents and Actions 8 27-34

AMSDEN, A.F. and BOROS, D.L. (1979) Fc receptor bearing macrophages isolated from hypersensitivity and foreign body granulomas. Am. J. Pathol. 96 457-473

AREND, W.P. and MANNIK, M. (1973) The macrophage receptor for IgG; number and affinity of binding sites. J. Immunol.110 1455-1463

ASHCROFT, T. and HEPPLESTON, A.G. (1973) Asbestos fibre concentration in relation to pulmonary reaction. in Bogovski et al Eds. 1973 236-237.

ATKINSON, J.P. and PARKER, C.W. (1977) Modulation of macrophage Fc receptor function by cytochalasin sensitive structures. Cell Immunol. 33 353-363

AXLINE, S.C. and REAVEN, E.P. (1974) Inhibition of phagocytosis and plasma membrane mobility of the cultivated macrophage by cytochalasin B. Role of subplasmalemmal microfilaments. J. Cell Biol. 62 647-659

BADR, F.M. and EL SEWEFY, A.Z. (1971) The association between asbestosis and ABO blood groups. Ann. occup. Hyg. 14 35-40

BAILEY, N.T.J. (1959) Statistical methods in Biology. English Univ. Press

BAINTON, D.F. (1980) The cells of inflammation: a general view. in Cell Biology of Inflammation. Ed. G. Weissman, Elsevier/North Holland 1-25

BALDWIN, R.W. (1976) Role of immunosurveillance against chemically induced rat tumours. Transplant Rev. 28 62-74

BARLIN, E., LESER, H.G., DEIMANN, W., TILL, G., RESCH, K. and GEMSA, D. (1981) In vivo activation of macrophages by Corynebacterium parvum, glucan and pyran co-polymer: different effects on prostaglandin E release, tumour cytostasis and lymphocyte mitogenicity. in Forster and Landy Eds. 1981, 243-252

BATEMAN, E., EMERSON, R. and COLE, E. (1980) The use of diffusion chambers to examine the biological effects of mineral dusts. in Brown et al Eds. 1980, 289-296

BECKLAKE, M. (1976) Asbestos related diseases of the lung and other organs. Their epidemiology and implications for clinical practice. Am. Rev. Resp. Dis. 114 187-227

BELLER, D.I., FARR, A.G. and UNANUE, E.R. (1978) Regulation of lymphocyte proliferation and differentiation by macrophages. Fed. Proc. 37 91-96

BELLER, D.I., and UNANUE, E.R. (1980) Thymocyte response to a growth promoting activity from macrophages: a dual signal model. in Biochemical Characterization of Lymphokines. Eds. De Weck, A.L., Kristensen, F. and Landy, M. Academic 427-431

BELLER, D.I. and UNANUE, E.R. (1981) Regulation of macrophage populations II Synthesis and expression of Ia antigens by peritoneal exudate macrophages is a transient event. J. Immunol. 126 263-269

BENOLIEL, A.M., CAPO, C., BONGRAND, P., RYTER, A. and DEPIEDS, R. (1980) Non-specific binding by macrophages - existence of different adhesive mechanisms and modulation by metabolic inhibitors. Immunology 41 547-560

BERRY, G. and WAGNER, J.C. (1976) Effect of age at inoculation of asbestos on occurrence of mesotheliomas in rats. Int. J. Cancer 17 447-483

BERRY, G. and LEWINSOHN, H.C. (1979) Dose response relationships for asbestos related disease: implications for hygiene standards. Part I Morbidity. Ann. N.Y. Acad. Sci. 330 185-194

BIANCO, C., EDEN, A. and COHN, Z.A. (1976) The induction of macrophage spreading: role of coagulation factors and the complement system. J. Exp. Med. 144 1531-1544

BIANCO, C. and EDELSON, P.J. (1978) Plasma membrane expressions of macrophage differentiation. Birth Defects: Original Artical Series XIV 119-124

BIANCO, C., GOTZE, O. and COHN, Z.A. (1980) Complement, coagulation and mononuclear phagocytes. in Mononuclear Phagocytes: Functional Aspects Vol. II. Ed. van Furth, R. Martinus Nijhoff 1443-1458

BITTERMAN, P., RENNARD, S., SCHOENBERGER, C. and CRYSTAL, R.(1981) Asbestos stimulates alveolar macrophages to release a factor causing human lung fibroblasts to replicate. Chest 80 38S-39S

BLYDEN, G., and HANDSCHUMACHER, P.E. (1977) Purification and properties of human lymphocyte activating factor (LAF). J. Immunol.118 1631-1638

BOGOVSKI, P., TIMBRELL, V., GILSON, J.C. and WAGNER, J.C. Eds. (1973) Biological Effects of Asbestos. IARC Pub. No. 8

BOLTZ-NITELESCU, G. and SPIEGELBERG, H.L. (1981) Receptors specific for IgE on rat alveolar and peritoneal macrophages. Cell Immunol. 59 106-114

BONNEY, R.J., DAVIES, P., KUEHL, F.A. and HUMES, J.L. (1981) Arachidonic acid oxygenation by various populations of macrophages. in Forster and Landy Eds. 1981, 257-262

BOROS, D. (1981) The role of lymphokines in granulomatous inflammation. in Lymphokines Vol. III Lymphokine and macrophage activation. Ed. Pick, E. Academic Press 257-281

BOWEN, J.G. and KULATILAKE, A.E. (1979) A common marker for cancer cells? Br. J. Cancer 40 806-808

BRAENDSTRUP, O., WERDELIN, O., SHEVACH, E.M. and ROSENTHAL, A.S. (1979) Macrophage lymphocyte clusters in the immune response to soluble protein antigen in vitro: VII Genetically restricted and non-restricted physical interactions. J. Immunol. 122 1608-1613

BRAIN, J.D. (1980) Macrophage damage in relation to the pathogenesis of lung disease. Env. Health Perspect 35 21-28

BRAMWELL, M.E. and HARRIS, H. (1978) An abnormal membrane glyco-protein associated with malignancy in a wide range of different tumours. Proc. R. Soc. Lond. B. 201 87

BRAND, K.G., JOHNSON, K.H. and BUONEN, L.C. (1976) Foreign Body Tumorigenesis. CRC Crit. Rev. Toxicol. 4353-394

BRODY, A.R. and HILL, L.H. (1981) Deposition pattern and clearance pathways of inhaled chrysotile asbestos. Chest 80 64S-67S

BROWN, D.G., WAGNER, J.C. and WAGNER, M.M.F. (1980) Failure to demonstrate tumour associated transplantation antigens in asbestos induced mesotheliomas in rats. Br. J. Cancer 42 797-801

BROWN, J.P., TAMERIUS, J.D. and HELLSTRÖM, I. (1979) Indirect [^{125}I] Protein A assay for monoclonal antibodies to cell surface antigens. J. Immunol. Methods 31 201-209

BROWN, R.C., CHAMBERLAIN, M., GRIFFITHS, D.M. and TIMBRELL, V. (1978) The effect of fibre size on the in vitro biological activity of three types of amphibole asbestos. Int. J. Cancer 22 721-727

BROWN, R.C., GORMLEY, I.P., CHAMBERLAIN, M. and DAVIES, R. (Eds.) (1980) In vitro effects of mineral dusts. Academic Press

BRUNE, K. (1980) Hepes buffered media may induce prostaglandin release from macrophages in tissue culture. Agents and Actions 10 491-492

BUCHANAN, W.D. (1965) Asbestosis and primary intrathoracic neoplasms. Ann. N.Y. Acad. Sci. 132 507-518

BURGER, M. (1970) Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. Nature 227 170-171

CALDERON, J. and UNANUE, E.R. (1975) Two biological activities regulating cell proliferation found in cultures of peritoneal exudate cells. Nature 253 359-361

CALDERON, J., WILLIAMS, R.T. and UNANUE, E.R. (1974) An inhibitor of cell proliferation released by cultured macrophages. Proc. Nat. Acad. Sci. 71 4273-4277

CALDERON, J., KIELY, J.M., LEFKO, J.L. and UNANUE, E.R. (1975) The modulation of lymphocyte function by molecules secreted by macrophages: I. disruption and partial biochemical analysis. J. Exp. Med. 142 151-164

CATHCART, M.K. and KRAKAUER, R.S. (1980) Abnormal immunoregulation of collagen accumulation in progressive systemic sclerosis. in Immunoregulation and autoimmunity. Eds. Krakauer, R.S. and Cathcart, M.K. Elsevier/North Holland 165-173

CHABOT, J.F., BEARD, D., LANGLOIS, A.J. and BEARD, J.W. (1970) Mesothelioma of peritoneum, epicardium and pericardium induced by strain MC29 avian leukosis virus. Cancer Res. 30 1287-1308

CHAMBERLAIN, M., and TARMY, E.M. (1977) Asbestos and glass fibres in bacterial mutation tests. Mut. Res. 43 159-164

CHAMBERLAIN, M. and BROWN, R.C. (1978) The cytotoxic effects of asbestos and other mineral dust on tissue culture cell lines. Br. J. Exp. Pathol. 59 183-189

CHAMPNESS, P.E., CLIFF, G. and LORIMER, G.W. (1976) The identification of asbestos. J. Microscopy 108 231-249

CHEN, P.S., TORIBARA, T.Y. and WARNER, H. (1956) Microdetermination of phosphorus. Anal. Chemistry 28 1756-1758

CHEUNG, H.T., CANTARON, W.D. and SUNDHARADAS, G. (1978) Colchicine and cytochalasin B (CB) effects on random movement, spreading and adhesion of mouse macrophages. Exp. Cell Res. 111 95-103

CHOWDHURY, S. (1975) Kinetics of leaching of asbestos mineral at body temperature. J. appl. Chem. Biotechnol. 25 347-353

COHN, Z.A. (1978) Macrophage activation: Fact fancy and future. J. Immunol. 121 813-816

COHN, Z.A. and BENSON, B. (1965) The differentiation of mononuclear phagocytes. Morphology, cytochemistry and biochemistry. J. Exp. Med. 121 153-170

COLE, D.A., STEVENS, R.H. and WILL, L.A. (1982) Identification of carcinogens by measurement of cell mediated immunity. III Immunity to asbestos induced rat peritoneal mesothelioma. Env. Res. 28 77-83

COOPER, W.C. (1978) Epidemiological evidence of the effect of type of asbestos and fiber dimension on the production of disease in man. in Gravatt et al Eds. 1977, 121-131

CRAIGHEAD, J.E., MOSSMAN, B.T. and BRADLEY, B.J. (1980) Comparative studies on the cytotoxicity of amphibole and serpentine asbestos. Env. Health Perspect. 34 37-46

CRAIGHEAD, J.E. and MOSSMAN, B.T. (1982) The pathogenesis of asbestos associated diseases. New Eng. J. Med. 306 1446-1455

CRAWFORD, O. and MILLER, K. (1981) Structure and structural changes in crocidolite asbestos associated with biological systems. Micron 12 25-28

CUNNINGHAM, B.A., SELA, B-A, ICHIRO, Y. and EDELMAN, G.M. (1976) Structure and activities of lymphocyte mitogens. in Oppenheim and Rosenstreich, Eds. 1976, 13-30

CUNNINGHAM, H.M. and PONTEFRACT, R. (1971) Asbestos fibres in beverages and drinking water. Nature (London) 232 332-333

CURRIE, G.A. (1981) Promotion of fibrosarcoma cell growth by products of syngeneic host macrophages. Br. J. Cancer 44 506-513

DANIEL, F.B., BEACH, C.A. and HART, R.W. (1980) Asbestos induced changes in the metabolism of polycyclic aromatic hydrocarbons in human fibroblast cell cultures. in Brown et al Eds. 1980, 255-262

DAVID, J.R. and REMOLD, H.G. (1976) Macrophage activation by lymphocyte mediators and studies on the interactions of macrophage inhibiting factor with its target cell. in Nelson, D.S. Ed. 1976, 401-426

DAVID, J.R., PIESSENS, W.F. and CHURCHILL, W.H. (1976) Macrophage activation by lymphocyte mediators and tumor immunity: a brief review. in Fink, M. Ed. 1976, 67-75

DAVIS, J.M.G. (1965) Electron microscopic studies of asbestosis in man and animals. Ann. N.Y. Acad. Sci. 132 98-111

DAVIS, J.M.G. (1970a) Further observations on the ultrastructure and chemistry of the formation of asbestos bodies. Exp. Mol. Pathol. 13 346-358

DAVIS, J.M.G. (1970b) The long term fibrogenic effects of chrysotile and crocidolite asbestos injected into the pleural cavity of experimental animals. Br. J. Exp. Path. 51 617-627

DAVIS, J.M.G. (1972a) The fibrogenic effects of mineral dusts injected into the pleural cavity of mice. Br. J. Exp. Path. 53 190-204

DAVIS, J.M.G. (1972b) The effects of polyvinyl pyridine-n-oxide (P204) on the cytopathogenic action of chrysotile asbestos in vivo and in vitro. Br. J. Exp. Path 53 652-658

DAVIS, J.M.G., (1974a) Ultrastructure of human mesotheliomas. J. Nat. Cancer Inst. 52 1715-1725

DAVIS, J.M.G. (1974b) Histogenesis and fine structure of peritoneal tumours produced in animals by injection of asbestos. J. Nat. Cancer Inst. 52 1823-1837

DAVIS, J.M.G. and CONIAM, S.W. (1973) Experimental studies on the effects of heated chrysotile asbestos and automobile brake lining dust injected into the body cavities of mice. Exp. Mol. Pathol. 19 339-353

DAVIS, J.M.G. and GROSS, P. (1973) Are ferruginous bodies an indication of atmospheric pollution by asbestos. in Bogovski et al Eds. 1973, 238-242

DAVIS, J.M.G., BECKETT, S.T., BOLTON, R.E., COLLINGS, P. and MIDDLETON, A.P. (1978) Mass and number of fibres in the pathogenesis of asbestos related lung disease in rats. Br. J. Cancer 37 673-688

DAVIES, P., ALLISON, A.C., ACKERMAN, J., BUTTERFIELD, A. and WILLIAMS, S. (1974) Asbestos induces selective release of lysosomal enzymes from mononuclear phagocytes. Nature 251 423-425

DAVIES, P. and ALLISON A.C. (1976) Secretion of macrophage enzymes in relation to the pathogenesis of chronic inflammation. in Nelson, D.S. Ed. 1976, 427-461

DAVIES, P and BONNEY, R.J. (1980) The secretion of hydrolytic enzymes by mononuclear phagocytes. in The cell biology of Inflammation, Handbook of Inflammation Vol. 2. Ed. Weissmann G. 497-542

DAVIES, W.A. and STOSSEL, T.P. (1981) External membrane proteins of rabbit lung macrophages. Arch. Biochem. Biophys 206 190-197

DE BAKER, J.M. and DAEMS, W.Th.(1981) The heterogeneity of mouse peritoneal macrophages. in Forster and Landy, Eds. 1981, 11-13

DE LUSTRO, F., SHERER, G.K. and LE ROY, G.C. (980) Human monocyte stimulation of fibroblast growth by a soluble mediator. J. Reticuloendothel. Soc. 28 519-532

DE PETRIS, S. (1974) Inhibition and reversal of capping by cytochalasin B, vinblastine and colchicine. Nature 250 54-56

DE WATER, R., van't NOORDENDE, J.M., GINSEL, L.A. and DAEMS, W.Th. (1981) Heterogeneity in wheat germ agglutinin binding by mouse peritoneal macrophages. Histochem. 72 333-339

DHEW NIOSH (1976) Revised recommended asbestos standard. DHEW (NIOSH) Pubn. 77-169

DIAMANSTEIN, T., HANDSCHUMAKER, R.E., OPPENHEIM, J.J., ROSENSTREICH, D.L., UNANUE, E.R., WAKSMAN, B.H. and WOOD, D.D. (1979) Non-specific lymphocyte activating factors produced by macrophages. J. Immunol. 122 2633-2635

DIMITRIOU, A., DY, M., THOMSON, N. and HAMBURGER, J. (1975) Macrophage cytotoxicity in the mouse immune response against a skin allograft. J. Immunol 114 195-199

DOLL, R. (1955) Mortality from lung cancer in asbestos workers. Br. J. Indust. Med. 12 81-86

DRESSER, D.W. (1978) Most IgM secreting cells in the mouse secrete auto-antibodies (rheumatoid factor). Nature 274 480-483

ECONOMOU, J.S., and SHIN, H.S. (1980) Purification, physicochemical characterization and a biological role of lymphocyte activating factor (LAF). in Biochemical Characterization of Lymphokines Eds. de Weck, A., Kristensen, F. and Landy, M. Academic 419-421

EDELSON, P.J. (1980) Monocytes and macrophages: aspects of their cell biology. in Handbook of Inflammation, 2. The Cell Biology of Inflammation. Eds. Glynn, L.E., Houck, J.C. and Weissman, G. Elsevier/North Holland 469-495

EDGE, J.R. (1976) Asbestos related disease in Barrow-in-Furness. Env. Res. 11 244-247

EGUCHI, M., SANNES, P.L. and SPICER, S.S. (1979) Alterations in ultrastructural cytochemistry of complex carbohydrates of rat peritoneal macrophages during phagocytosis. J. Reticuloendothel. Soc. 25 207-218

ELMES, P.C. (1981) Relative importance of cigarette smoking in occupational lung disease. Br. J. Ind. Med. 38 1-13

EL SEWEFY, A.Z. and HASSAN, F. (1971) Immunoelectrophoretic pattern changes in asbestosis. Ann. occup. Hyg. 14 25-28

EL SEWEFY, A.Z., SHAHEEN, H. and SHAMS-EL-DEEN, A. (1974) Bone marrow changes in asbestosis. Med. Lav. 65 168-173

EMBLETON, M.J., WAGNER, J.C., WAGNER, M.M.F., JONES, J.S.P., SHEARS, G., OLDHAM, P.D. and BALDWIN, R.W. (1976) Assessment of cell mediated immunity to malignant mesothelioma by microcytotoxicity tests. Int. J. Cancer 17 597-601

ENTICKNAP, J.B. and SMITHERS, W.J. (1964) Peritoneal tumours in asbestosis. Br. J. Ind. Med. 21 20-31

ERB, P. and FELDMANN, M. (1975) The role of macrophages in the generation of T helper cells: I The requirements for macrophages in helper cell induction and characteristics of the macrophage T cell interaction. Cell. Immunol. 19 356-367

ERB, P., FELDMANN, M. and HOGG, N. (1976) Role of macrophages in the generation of T helper cells. IV Nature of genetically related factor derived from macrophages incubated with soluble antigens. Eur. J. Immunol. 6 365-372

EVANS, C.C., LEWINSOHN, H.C. and EVANS, J.M. (1977) Frequency of HLA antigens in asbestos workers with and without asbestosis. Br. Med. J. i 603-605

EVANS, M.J., CABRAL, L.J., STEPHENS, R.J. and FREEMAN, G. (1973) Cell division of alveolar macrophages in rat lung following exposure to NO_2. Am. J. Path. 70 199-208

EVANS, R. (1976) Tumor macrophages in host immunity to malignancies. in Fink, M.A. Ed. 1976, 27-42

EVANS, R. (1979) Host cells in transplanted murine tumours and their possible relevance to tumour growths. J. Reticuloendothel. Soc. 26 427-437

EVANS, R. and ALEXANDER, P. (1972) Role of macrophages in tumour immunity: II Involvement of a macrophage cytophilic factor during syngeneic tumour growth inhibition. Immunology 23 627-636

EVANS, R., GRANT, C.K., COX, H., STEELE, K. and ALEXANDER, P. (1972) Thymus derived lymphocytes produce an immunologically specific macrophage arming factor. J. Exp. Med. 136 1318-1322

EVANS, R. and ALEXANDER, P. (1976) Mechanisms of extracellular killing of nucleated mammalian cells by macrophages. in Nelson, D.S. Ed. 1976, 535-576

FARRAM, E. and NELSON, D.S. (1980) Mechanism of action of mouse macrophages as anti-tumour effector cells: role of arginase. Cell. Immunol. 55 283-293

FAUVE, R.M., HEVIN, M.B. and DAUGE, M.C. (1977) Inflammation and activation of macrophages. in James et al Eds. 1977, 258-263.

FERLUGA, J., SCHORLEMMER, H.U., BAPTISTA, L.C. and ALLISON, A.C. (1978) Production of the complement cleavage product C3a by activated macrophages and its tumorolytic effect. Clin. Exp. Immunol. 31 512-517

FINK, M.A.(Ed.) (1976) The macrophage in neoplasia. Academic Press

FORSTER, O and LANDY, M. (Eds.) (1981) Heterogeneity of Mononuclear Phagocytes. Academic Press.

FRANK, A.L. (1980) Asbestos induced changes in hamster trachea organ culture. in Brown et al Eds. 1980, 235-240

FRANTZ, C.N., STILES, C.D. and SCHER, C.D. (1979) The tumour promoter 12-o-tetradecanoyl phorbol 13 acetate enhances the proliferative response of Balb/C 3T3 cells to hormonal growth factors. J. Cell. Physiol 100 413-424

GABIZON, A. and TRAININ, N. (1980) Enhancement of tumour growth by peritoneal macrophages of normal and tumour bearing mice. in Macrophage and Lymphocytes: Nature, Function and Interactions. Part B. Eds. Escobar, M.R. and Friedman, H. Plenum 485-491

GALINDO, B., LAZDINS, J. and CASTILLO, R. (1974) Fusion of normal rabbit alveolar macrophages induced by supernatant fluids from BCG sensitized lymph node cells after elicitation by antigen. Infect. Immun. 9 212-228

GARRIDO, J., BURGLEN, M.J., SAMOLYK, D., WICKER, R. and BERNHARD, W. (1974) Ultrastructural comparison between distribution of Concanavalin A and wheat germ agglutinin cell surface receptors of normal and transformed hamster and rat cell lines. Cancer Res. 34 230-243

GAUMER, H.R., DOLL, N.J., KAIMAL, J., SCHUYLER, M. and SALVAGGIO, J.E. (1981) Diminished suppressor cell functions in patients with asbestosis. Clin. exp. Immunol. 44 108-116

GAZE, R. (1965) The physical and molecular structure of asbestos. Ann. N.Y. Acad. Sci. 132 23-30

GERBER, M.A. (1970) Asbestosis and neoplastic disorders of the haematopoetic system. Am. J. Clin. Pathol. 53 204-208

GERY, I., GERSHON, R.K. and WAKSMAN, B.H. (1972) Potentiation of the T lymphocyte response to mitogens: I The responding cell. J. Exp. Med. 136 128-142

GERY, I. and WAKSMAN, B.H. (1972) Potentiation of the T lymphocyte response to mitogens: II cellular source of potentiating mediator(s). J. Exp. Med. 136 143-155

GILSON, J.C. (1965) Man and asbestos. Ann. N.Y. Acad Sci. 132 9-30

GLENN, K.C. and ROSS, R. (1981) Human monocyte-derived growth factor(s) for mesenchymal cells: activation of secretion by endotoxin and Concanavalin A. Cell 25 603-615

GOLDENBERG, D.M., SHARKEY, R.M. and PRIMUS, F.J. (1978) Immunocytochemical detection of carcino-embryonic antigen in conventional histopathology specimens. Cancer 42 1546-1553

GOLDSTEIN, B.D., HAMBURGER, S.J., FALK, G.W. and AMORUSO, M.A. (1977) Effect of ozone and nitrogen dioxide on the agglutination of rat alveolar macrophages by Concanavalin A. Life Sciences 21 1637-1644

GOLDSTEIN, J.C., ANDERSON, R.G.W. and BROWN, M.S. (1979) Coated pits coated vesicles and receptor mediated endocytosis. Nature 279 679-680

GOODWIN, J.S., BANKHURST, A.D. and MEISSNER, R.P. (1977) Suppression of human T cell mitogenesis by prostaglandins. Existence of prostaglandin producing suppressor cells. J. Exp. Med. 146 1719-1734

GORDON, S., UNKELESS, J.C. and COHN, Z.A. (1974) Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis: evidence for a two stage process. J. Exp. Med. 140 995-1010

GORMLEY, I.P., WRIGHT, A., COLLINGS, P. and DAVIS, J.M.G. (1980a) The cytotoxicity of UICC and modified asbestos fibres in vitro. in Biological Effects of Mineral Fibres. Ed. Wagner, J.C. IARC Pub. N. 30. Lyon 1980, 427-436

GORMLEY, I.P., BOLTON, R.E., BROWN, G., DAVIS, J.M.G. and DONALDSON, K. (1980b) Studies on the morphological pattern of asbestos induced mesotheliomas in vivo and in vitro. Carcinogenesis 2 219-231

GOTZE, O., BIANCO, C., SUNDSMO, J.S. and COHN, Z.A. (1980) The stimulation of mononuclear phagocytosis by components of the classical and the alternative pathways of complement activation. in Mononuclear Phagocytes: Functional Aspects. Part II. Ed. van Furth, R. Martinus Nijhoff 1421-1442

GRAVATT, C.C., LA FLEUR, P.D. and HEINREICH, F.J. (Eds) (1978) Workshop on asbestos: Definition and measurements. Gaithersburg, Maryland 1977. Proceedings

GRIFFIN, F.M. and SILVERSTEIN, S.C. (1974) Segmental response of the macrophage plasma membrane to a phagocytic stimulus. J. Exp. Med. 139 323-336

GRIFFIN, F.M., GRIFFIN, J.A., LEIDER, J.E. and SILVERSTEIN, S.C. (1975a) Studies on the mechanism of phagocytosis: I requirement for circumferential attachment of particles bound ligands to specific receptors on the macrophage plasma membrane. J. Exp. Med. 142 1263-1282

GRIFFIN, F.M., BIANCO, C. and SILVERSTEIN, S.C. (1975b) Characterization of the macrophage receptor for complement and its functional independence from the receptor for the Fc portion of Immunoglobulin G. J. Exp. Med. 141 1269-1277

GROSS, P., DE TREVILLE, R.T.P., TOLKER, E.D., KOSCHEK, M. and BABYAT, M.A. (1967) Experimental asbestosis. The development of lung cancer in rats with pulmonary deposits of chrysotile asbestos dust. Arch. Environ. Health 15 343-355

HADLEY, J.G., GARDNER, D.C., COFFIN. O.L. and MENZEL, D.B. (1977) Effect of ozone and nitrogen dioxide on the agglutination of rat alveolar macrophages by Concanavalin A. Life Sciences 21 1637-1644

HAHON, N. and ECKERT, H.L. (1976) Depression of viral interferon induction in cell monolayers by asbestos fibres. Env. Res. 11 52-65

HAMILTON, J.A. (1980) Macrophage stimulation and the inflammatory response to asbestos. Env. Health Perspect. 34 69-74

HAMILTON, J.A., VASSALLI, J-D, and REICH, E. (1976) Macrophage plasminogen activator: induction by asbestos is blocked by anti-inflammatory steroids. J. Exp. Med. 144 1689-1694

HAMILTON, J.A., CHAN, J.Y. and MOVAT, H.Z. (1981) Coagulation of human plasma by asbestos fibres. Env. Res. 26 119-124

HAMMOND, E.C., SELIKOFF, I.J. and SEIDMAN, H. (1979) Asbestos exposure, cigarette smoking and death rates. N.Y. Acad. Sci. 330 473-490

HAMMOND, M.E. and DVORAK, H.F. (1972) Antigen induced stimulation of glucosamine incorporation by guinea pig peritoneal macrophages in delayed hypersensitivity. J. Exp. Med. 136 1518-1532

HANNA, M.G., BUCANA, C., HOBBS, B. and FIDLER, I.J. (1976) Morphological aspects of tumour cell cytotoxicity by effector cells of the macrophage histocyte compartment: in vitro and in vivo studies in BCG mediated tumour regression. in Fink, M., Ed. 1976, 113-133

HARINGTON, J.S. (1965) Chemical Studies of asbestos. Ann. N.Y. Acad. Sci. 132 31-47

HARINGTON, J.S. (1974) Fibrogenesis. Env. Health Perspect. 9 271-279

HARINGTON, J.S. (1976) The biological effects of mineral fibres especially asbestos as seen from in vitro and in vivo studies. Ann. Anat. Pathol. 21 155-198

HARINGTON, J.S., MILLER,K., MACNAB, G. (1971) Hemolysis by asbestos. Env. Res. 4 95-117

HARINGTON, J.S., ALLISON, A.C. and BADAMI, D.V. (1975) Mineral Fibres: chemical, physicochemical and biological properties. Adv. Pharmacol. and Chemotherapy 12 291-402

HARRIES, P.G. (1968) Asbestos hazard in a naval dockyard. Ann. occup. Hyg. 11 135-145

HARRIS, R.L. and FRASER, D.A. (1976) A model for deposition of fibres in the human respiratory system. Am. Ind. Hyg. J. 37 73-89

HART, R.W., KENDY, O., BLAKESLEE, J. and MIZUHIRA, V. (1980) Effects of cellular ingestion on the elemental ratio of asbestos. in Brown et al Eds. 1980, 191-198

HASLAM, P.L., LUKOSZEK, A., MERCHANT, J.A. and TURNER-WARWICK, M. (1978) Lymphocyte responses to phytohaemagglutinin in patients with asbestosis and mesothelioma. Clin. Exp. Immunol. 31 178-188

HASSELBACHER, P. (1979) Binding of immunoglobulin and activation of complement by asbestos fibres. J. Allergy Clin. Immunol. 64 294-298

HENDRY, N.W. (1965) The geology, occurrence and major uses of asbestos. Ann. N.Y. Acad. Sci. 132 9-30

HEPPLESTON, A.G. (1982) Silicotic fibrogenesis: A concept of pulmonary fibrosis. Inhaled Particles V In press.

HEPPLESTON, A.G. and STYLES, J.A. (1967) Activity of macrophage factor in collagen formation by silica. Nature 214 521-522

HERBERMAN, R.B. (1978) In vitro tests of cellular immunity in man. Invest. Cell. Pathol. 1 227-248

HERBERMAN, R.B., HOLDEN, H.T., DJEV, J.Y., JERRELLS, T.R., VARESIO, L., TAGLIABUE, A., WHITE, S.L., OEHLER, J.R. and DEAN, J.H. (1980) Macrophages as regulators of immune responses against tumours. in Macrophages and Lymphocytes: Nature, Functions and Interactions. Part B. Eds. Escobar, M.R. and Friedman, H. Plenum 361-379

HEXT, P.M., HUNT, J., DODGSON, K.S. and RICHARDS, R.J. (1977) The effects of long term exposure of lung fibroblast strains to chrysotile asbestos. Br. J. Exp. Path. 58 160-167

HIBBS, J.B. (1972) Control of carcinogenesis - A possible role for the activated macrophage. Science 177 998-1000

HIBBS, J.B. (1974) Heterocyclosis by macrophages activated by Bacillus Calmette Guerin: lysosome exocytosis into tumour cells. Science 184 468-471

HIBBS, J.B., CHAPMAN, H.A. and BRICE-WEINBERG, J. (1978) The macrophage as an antineoplastic surveillance cell: biological perspectives. J. Reticuloendothel. Soc. 5 549-570

HIBBS, J.B., WEINBERG, J.B. and CHAPMAN, H.A. (1980) Modulation of the tumoricidal function of activated macrophages by bacterial endotoxin and mammalian macrophage activation factor(s). in Macrophage and Lymphocytes: Nature, Functions and Interactions. Part B. Eds. Escobar, M.R. and Friedman, H. Plenum 433-453

HIBBS, J.B. and GRANGER, D.L. (1981) Activated macrophage induced respiratory inhibition in neoplastic cells: identification of lytic and non-lytic neoplastic cell types. in Forster and Landy, Eds. 1981, 477-483

HIRSCH, M.S., GARY, G.W. and MURPHY, F.A. (1969) In vitro and in vivo properties of anti-macrophage serum. J. Immunol. 102 656-661

HODGSON, A.A. (1965) Fibrous silicates. Royal Institute of Chemistry Lecture Series, No. 4 1-46

HODGSON, A.A. (1975) Asbestos - a world resource: 2. Amphibole asbestos. in III Int. Conference on the physics and chemistry of asbestos. Quebec Abstracts. Abstract 1.2 10 pp.

HOFFMANN, M.K., KOENIG, S., MITTLER, R.S., OETTGEN, H.F., RALPH, P., GALANOS, C., and HAMMERLING, U. (1979) Macrophage factor controlling differentiation of B cells. J. Immunol. 122 497-502

HOLT, P.F., MILLS, J. and YOUNK, D.K. (1965) Experimental asbestosis with four types of fibre: importance of small particles. Ann. N.Y. Acad. Sci. 132 87-97

HOLT, P.G. (1979) Alveolar macrophages. II Inhibition of lymphocyte proliferation by purified macrophages from rat lung. Immunology 37 429-436

HOLT, P.G., WARNER, L.A. and MAYRHOFER, G. (1981) Macrophages as effectors of T suppression: T lymphocyte dependent macrophage mediated suppression of mitogen induced blastogenesis in the rat. Cell. Immunol. 63 57-70

HOMMA, Y., ONOZAKI, K., HASHIMOTO, T., MIURA, K., NAGAO, S. and TANAKA, A. (1981) Different effect of (L) fucose binding lectin on macrophage migration inhibition caused by guinea pig migration inhibition factor and synthetic muramyl dipeptide. Int. Arch. Allerg. Appl. Immunol. 65 27-33

HOPPER, K.E., WOOD, P.R. and NELSON, D.S. (1979) Macrophage Heterogeneity. Vox. Sang. 36 257-274

HOPPER, K.E., GECZY, C.L. and DAVIES, W.A. (1981) A mechanism of migration inhibition in delayed type hypersensitivity reactions: fibrin deposition on the surfaces of elicited peritoneal macrophages in vivo. J. Immunol. 126 1052-1058

HOWIE, S. and FELDMANN, M. (1978) Immune response (Ir) genes expressed at macrophage B lymphocyte interactions. Nature 273 664-666

HUANG, S.L. (1979) Amosite, chrysotile and crocidolite are mutagenic in Chinese hamster lung cells. Mut. Res. 68 265-274

HUMES, J.L., BONNEY, R.J., PELUS, L., DAHLGREN, M.E., SADOWSKI, S.J., KUEHL, F.A. and DAVIES, P. (1977) Macrophages synthesize and release prostaglandins in response to inflammatory stimuli. Nature 269 149-151

HUMPHREY, E.W., EWING, S.L., WRIGLEY, J.V., NORTHRUP, W.F., KERSTEN, T.E., MAYER, J.E. and VARCO, R.L. (1981) The production of malignant tumours of the lung and pleura in dogs from intratracheal asbestos instillation and cigarette smoking. Cancer 47 1994-1999

HUNTER, W.M. (1978) Radioimmunoassay. in Weir, D.M. (Ed.) Handbook of Experimental Immunology. Blackwell, 14.1-14.40

HUUSKONEN, M.S., RASANEN, J.A., HARKONEN, H. and ASP, S. (1978) Asbestos exposure as a cause of immunological stimulation. Scand. J. Resp. Dis. 59 326-332

ISA, A.M. (1974) In vitro specificity and in vivo activity of anti-macrophage serum. Transplantation 17 276-280

JAMES, K. (1980) Alpha-2-macroglobulin and its possible importance in immune systems. Trends in Biochemical Science 5 43-47

JAMES, K., McBRIDE, B. and STUART, A.E. (Eds.) (1977) The macrophage and cancer. The Editors.

JAURAND, M.C., MAGNE, L. and BIGNON, J. (1979a) Inhibition by phospholipids of haemolytic action of asbestos. Br. J. Industr. Med. 36 113-116

JAURAND, M.C., KAPLAN, H., THIOLLET, J., PINCHON, M.C., BERNAUDIN, J.F. and BIGNON, J. (1979b) Phagocytosis of chrysotile fibres by pleural mesothelial cells in culture. Am. J. Path. 94 529-532

JAURAND, M.C., MAGNE, L. and BIGNON, J. (1980) The significance of lysosomal enzyme release induced by asbestos. in Brown et al Eds. 1980, 83-87

JOHNSON, R.L. and ZIFF, M. (1976) Lymphokine stimulation of collagen accumulation. J. Clin. Invest. 58 240-252

JOHNSTON, R.B., CHADWICK, D.A. and COHN, Z.A. (1981) Priming of macrophages for enhanced oxidative metabolism by exposure to proteolytic enzymes. J. Exp. Med. 153 1678-1683

JONES, T.C. (1975) Attachment and ingestion phases of phagocytosis. in Mononuclear Phagocytes in Immunity, Infection and Pathology. Ed. van Furth, R. Blackwell, 269-282

KAGAMIMORI, S., SCOTT, M.P., BROWN, D.G., EDWARDS, R.E. and WAGNER, M.M.F. (1980) Effects of chrysotile asbestos on mononuclear cells in vitro. Br. J. Exp. Pathol. 61 55-60

KAGAN, E. (1980) The alveolar macrophages: immune derangement and asbestos related malginancy. Sem. Onc. 8 258-267

KAGAN, E., SOLOMON, A., COCHRANE, J.C., BRESSNER, E.I., GLUCKMAN, J., ROCKS, P.H. and WEBSTER, I. (1977a) Immunological studies of patients with asbestosis: I studies of cell mediated immunity. Clin. exp. Immunol. 28 261-267

KAGAN, E., SOLOMON, A., COCHRANE, J.C., KUBA, P., ROCKS, P.H. and WEBSTER, I. (1977b) Immunological studies in patients with asbestosis: II studies of circulating lymphoid cell numbers and humoral immunity. Clin. exp. Immunol. 28 268-275

KAGAN, E. and MILLER, K. (1981) Asbestos inhalation and the production of splenic lymphocytic proliferation in the rat. Chest 80 115-125

KAKUNAGA, T. (1975) The role of cell division in the malignant transformation of mouse cells treated with 3 methyl-cholanthrene. Cancer Res. 35 1637-1642

KANAZAWA, K., YAMAMOTO, T., and YUASA, Y. (1979) Enhancement by asbestos of oncogenesis by Moloney murine sarcoma virus in CBA mice. Int. J. Cancer 23 866-874

KANDASWAMI, C. and O'BRIEN, J.J. (1980) Effects of asbestos on membrane transport and metabolism of benzo(∝) pyrene. Biochem. Biophys. Res. Comm. 97 794-801

KANE, A.B., STANTON, R.P., RAYMOND, E.G., DOBSON, M.E., KNAFELC, M.E. and FARBER, J.L. (1980) Dissociation of intracellular lysosomal rupture from cell death caused by silica. J. Cell. Biol. 87 643-651

KANG, K.Y., SERA, Y., OKOCHI, T. and YAMAMURA, Y. (1974) T lymphocytes in asbestosis. N. Engl. J. Med. 291 735-736

KANNERSTEIN, M., CHURG, J. and McCAUGHEY, W.T.E. (1978) Asbestos and mesothelioma: a review. Pathology Annual 13 81-128

KAPLAN, A.M. and MOHANAKUMAR, T. (1977) Expression of a new cell surface antigen on activated macrophages. J. Exp. Med. 146 1461-1466

KAPLAN, G. and SELJELID, R. (1977) Striking differences in interaction between macrophages and two closely related tumour lines. in James et al Eds. 1977, 347-363

KAPLAN, G. and OLSTAD, R. (1981) Heterogeneity in surface glycoproteins of mouse peritoneal macrophage populations. Exp. Cell. Res. 135 379-386

KAPLAN, H., JAURAND, M.C., PINCHON, M.C., BERNAUDIN, J.F. and BIGNON, J. (1980a) How pleural mesothelial cells react in vitro with chrysotile fibres. in Biological Effects of Mineral Fibres. Ed. Wagner, J.C. IARC Pub. No. 3, Lyon 451-457

KAPLAN, H., RENIER, M.C., JAURAND, M.C. and BIGNON, J. (1980b) Sister chromatid exchanges in mesothelial cells cultured with chrysotile fibres. in Brown et al Eds. 1980, 251-253

KARNOVSKY, M.C. and LAZDINS, J.K. (1978) Biochemical criteria for activated macrophages. J. Immunol. 121 809-813

KELLER, R. (1975) Cytostatic and cytocidal effects of activated non-immune macrophages. in Fink, M. Ed. 1976, 149-164

KELLER, R. (1976) Cytostatic and cytocidal effects of activated macrophages. in Nelson, D.S., Ed. 1976, 487-508

KELLER, R. (1977) Mononuclear phagocytes and anti-tumour resistance. in James et al Eds. 1977, 31-48

KELLER, R. (1978) Macrophage mediated natural cytotoxicity against various target cells in vitro: I Macrophages from diverse anatomical sites and different strains of rats and mice. Br. J. Cancer 37 732-746

KELLER, R. (1981) The cytostatic and cytocidal effects of macrophages: are they really specific for tumour cells. in Lymphokines, Vol. 3 Lymphokines and Macrophage Activation. Ed. Pick, E. Academic Press 283-292

KELLER, R., KEIST, R. and HECKER, E. (1982) Tumour promoting diterpene esters prevent macrophage activation. Br. J. Cancer 45 144-146

KING, E.J., CLEGG, J.W. and RAE, V.M. (1946) Effect of asbestos, and of asbestos and aluminium, on the lungs of rabbits. Thorax 1 188-197

KLIMPEL, G.R. and HENNEY, C. (1978) BCG induced suppressor cells. Demonstration of a macrophage-like suppressor cell that inhibits cytotoxic T cell generation in vitro. J. Immunol. 120 563-569

KLOSTERKOTTER, W. (1968) Experimentelle Untersuchungen uber die Bedeutung der Faserlange fur die Asbest fibrose sowie Untersuchungen uber die Beeinflussung der Fibrose durch Polyvinyl-pyridin-n-oxid. in Biologische Wirkungen des Asbestes Internationale Konferenz, 1968. Dresden Conference Reports, 1968.

KNIEP, E.M., DOMZIG, W., LOHMANN-MATHES, M.L. and KIRKHOFEN B. (1981) Particle purification and chemical characterization of macrophage cytotoxicity factor (MCF/MAF) and its separation from migration inhibitory factor (MIF). J. Immunol. 127 417-422

KOOPMAN, W.J., FARRAR, J.J. and FULLER-BONAR, J. (1978) Evidence for the identification of lymphocyte activating factor as the adherent cell derived mediator responsible for enhanced antibody synthesis by nude mouse spleen cells. Cell. Immunol. 35 92-98

KRAVIS, T.C., AHMED, A., BROWN, T.E., FULMER, J.D., and CRYSTAL, R.C. (1976) Pathogenic mechanisms in pulmonary fibrosis: collagen induced migration inhibition factor production and cytotoxicity mediated by lymphocytes. J. Clin. Invest. 58 1223-1232

KULONEN, E., AALTO, M., AHO, S., LEHTONEN, P. and POTILA, M. (1980) The SiO_2 liberated fibrogenic macrophage factors with reference to RNA. in The in vitro effects of mineral dusts. Brown et al Eds. 1980, 281-288

LAKOWICZ, J.R. and HYLDEN, J.L. (1978) Asbestos mediated membrane uptake of benzo (α) pyrene observed by fluorescence spectroscopy. Nature 275 446-448

LANGE, A. (1980) An epidemiological survey of immunological abnormalities in asbestos workers. I non-organ and organ specific auto-antibodies. II serum Ig levels. Env. Res. 22 176-183

LANGE, A., SMOLIK, R., CHMIELARCZYK, W., GARNCAREK, D. and GIELGIER, Z. (1978) Cellular immunity in asbestos. Arch. Immun. Therap. Exp. 26 899-903

LASKIN, D.L., LASKIN, J.D., WEINSTEIN, I.B. and CARCHMAN, R.A. (1980) Modulation of phagocytosis by tumour promoters and epidermal growth factors in normal and transformed macrophages. Cancer Res. 40 1028-1035

LAVAPPA, K.S., FU, M.M. and EPSTEIN, S.S. (1975) Cytogenetic studies on chrysotile asbestos. Env. Res. 10 165-173

LE BOUFFANT, L., DANIEL, H. and MARTIN, J.C. (1980) The value and limits of the relationship between cytotoxicity and fibrogenicity of various mineral dusts. in Brown et al Eds. 1980, 333-338

LEE, K.C. (1980) On the origin and mode of action of functionally distinct macrophage subpopulations. Mol. Cell. Biochem. 30 39-55

LEIBOVICH, S.J. (1978) Production of macrophage dependent fibroblast stimulating activity (M-FSA) by murine macrophages. Exp. Cell. Res. 13 47-56

LEIBOVICH, S.J. and ROSS,R. (1975) The role of the macrophage in wound repair: a study with hydrocortisone and anti-macrophage serum. Am. J. Pathol. 78 71-100

LEIBOVICH, S.J. and ROSS, R. (1976) A macrophage dependent factor stimulates the proliferation of fibroblasts in vitro. Am. J. Pathol. 84 501-513

LEIBOVICH, S.J. and DANON, D. (1980) Promotion of wound repair in mice by application of glucan. J. Reticuloendothel. Soc. 27 1-11

LEWINSOHN, H.C. (1974) Early malignant changes in pleural plaques due to asbestos exposure. Br. J. Dis. Chest 68 121-127

LIEBEN, J. (1966) Malignancies in asbestos workers. Arch. Env. Health 13 619-621

LIGHT, W.G. and WEI, E.T. (1977) Surface charge and asbestos toxicity. Nature 265 537-539

LINCOLN, B. (1975) Asbestos a world resource: I chrysotile. in III International Conference on the Physics and Chemistry of Asbestos. Quebec. Abstracts. Abstract 1.1 10pp.

LINDELL, K.V. (1973) Industrial uses of asbestos. in Bogovski et al Eds. 1973, 323-328

LIPSKY, P.E. and ROSENTHAL, A.S. (1975) Macrophage-lymphocyte interaction. II Antigen mediated physical interactions between immune guinea pig lymph node lymphocytes and syngeneic macrophages. J. Exp. Med. 141 138-154

LIVINGSTON, G.K., ROM, W.M. and MORRIS, M.V. (1980) Asbestos induced sister chromatid exchanges in cultured Chinese hamster ovarian fibroblast cells. J. Env. Pathol. Toxicol. 4 373-382

LOOR, F. and ROELANTS, G.E. (1974) The dynamic state of the macrophage plasma membrane attachment and fate of immunoglobulin antigen and lectins. Eur. J. Immunol. 4 649-660

LORIMER, W.V., ROHL, A.N., MILLER, A., NICHOLSON, W.J. and SELIKOFF, I.J. (1976) Asbestos exposure of brake repair workers in the United States. Mt. Sinai J. Med. 43 207-218

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951) Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193 265-275.

LUKENS, H.R. (1978) Production of antibody towards asbestos. Res. Communic. Chem. Pathol. Pharmacol. 21 517-522

LUTTON, J.D. (1973) The effect of phagocytosis and spreading on macrophage surface receptors for Concanavalin A. J. Cell. Biol. 56 611-617

McEWEN, J., FINLAYSON, A., MAIR, A. and GIBSON, A.A.M. (1970) Mesothelioma in Scotland. Br. J. Med. 4 575-578

MacKANESS, G.G. (1964) The immunological basis of acquired cellular resistance. J. Exp. Med. 120 105-120

MacNAB, C. and HARINGTON, J.J. (1967) Haemolytic activity of asbestos and other mineral dusts. Nature 214 522-523

McVITTIE, J.C. (1965) Asbestosis in Great Britain. Ann. N.Y. Acad. Sci. 132 128-138

MADDOX, D.E., SHIBATA, S. and GOLDSTEIN, I.J. (1982) Stimulated macrophages express a new glycoprotein receptor reactive with Griffonia simplicifolia I-B_4 isolation. Proc. N.Y. Acad. Sci. 79 166-170

MANTOVANI, A., RABINOVITCH, M. and NUSSENZWEIG, V. (1972) Phagocytosis of immune complexes by macrophages. Different roles of the macrophage receptor site for complement (C3) and for immunoglobulin (IgG). J. Exp. Med. 135 780-792

MANTOVANI, A., DEAN, J.H., JERRELLS, T.R. and HERBERMAN, R.B. (1980) Augmentation of tumoricidal activity of human monocytes and macrophages by lymphokine. Int. J. Cancer 25 691-699

MAROUDAS, N.G., O'NEIL, C.H. and STANTON, M.F. (1973) Fibroblast anchorage in carcinogenesis by fibres. Lancet i 807-809

MARQUES, E. (1978) Binding of Concanavalin A by normal and transformed cells and by their trypsin released surface components. Exp. Cell. Res. 115 143-149

MARTIN, B.M., GIMBRONE, M.A., UNANUE, E.R. and COTRAN, R.S. (1981) Stimulation of non-lymphoid mesenchymal cell proliferation by macrophage derived growth factor. J. Immunol. 126 1510-1515

MELTZER, M.S., RUCO, L.P., BORASCHI, D. and NACY, C.A. (1979) Macrophage activation for tumour cytotoxicity: analysis of intermediary reactions. J. Reticuloendothel. Soc. 26 403-415

MERCHANT, J.A., KLOUDA, P.T. and SOUTAR, C.A. (1975) The HLA system in asbestos workers. Br. Med. J. i 189-191

MEREWETHER, E.R.A. and PRICE, C.W. (1930) Reports on effects of asbestos dust on lungs and dust suppression in the asbestos industry. HMSO London.

METCHNIKOFF, E. (1893) Immunity in Infective Diseases. Johnson Reprint Corporation. Reprinted 1968.

MICHAELS, L. and CHISSICK, S.S. (1979) Asbestos Vol. I. Properties, Applications and Hazards. Wiley.

MICHL, J. and SILVERSTEIN, S.C. (1978) Role of macrophage receptors in the ingestion phase of phagocytosis. Birth Defects: Original Article Series, XIV 99-117

MILLER, K. (1978) The effects of asbestos on macrophages. CRC Crit. Rev. Toxicol. 5 319-354

MILLER, K. (1979) Alterations in the surface related phenomena of alveolar macrophages following inhalation of crocidolite asbestos and quartz dusts: an overview. Env. Res. 20 162-182

MILLER, K. and KAGAN, E. (1976) In vivo effects of asbestos on macrophages: membrane structure and population characteristics of macrophages: a scanning electron microscope study. J. Reticuloendothel. Soc. 20 159-171

MILLER, K. and KAGAN, E. (1977) Immune adherence reactivity of rat alveolar macrophages following inhalation of crocidolite asbestos. Clin. exp. Immunol. 29 152-158

MILLER, K., WEBSTER, I., HANDFIELD, R.I.M. and SKIKNE, M.I. (1978) Ultrastructure of the lung in the rat following exposure to crocidolite asbestos and quartz. J. Pathol. 124 39-44

MILLER, K., WEINTRAUB, Z. and KAGAN, E. (1979) Manifestations of cellular immunity in the rat after prolonged asbestos inhalation. I Physical interaction between alveolar macrophages and splenic lymphocytes. J. Immunol. 123 1029-1038

MILLER, K. and KAGAN, E. (1981) Manifestations of cellular immunity in the rat after prolonged asbestos inhalation. II Alveolar macrophages induce splenic lymphocyte proliferation. Env. Res. 26 182-194

MISHELL, B. and SHIIGI, S.M. (1980) Selected methods in cellular immunology. Freeman and Co.

MITCHISON, N.A. (1969) The immunogenic capacity of antigen taken up by peritoneal macrophages. Immunology 16 1-14

MIZEL, S. (1979) Physicochemical characterization of lymphocyte activating factor (LAF). J. Immunol. 122 2167-2172

MONCHAUX, G., BIGNON, J., JAURAND, M.C., LAFUMA, J., SEBASTIEN, P., MASSE, R., HIRSCH, A. and GONI, J. (1981) Mesotheliomas in rats following inoculation with acid leached chrysotile asbestos and other minerals. Carcinogenesis 2 229-236

MOORE, K. and McBRIDE, W.H. (1980) The activation state of macrophage sub-populations from a mouse fibrosarcoma. Int. J. Cancer 26 609-615

MOORE, M. (1978) Antigens of experimentally induced neoplasms - a conspectus. in Immunological aspects of Cancer. Ed. Castro, J.E. MTP 15-50

MORAHAN, P.S. (1980) Macrophage nomenclature; where are we going? J. Reticuloendothel. Soc. 27 223-245

MORGAN, A. (1974) Adsorption of human serum albumen by asbestiform minerals and its application to the measurement of surface areas of dispersed samples of chrysotile. Env. Res. 7 330-341

MORGAN, A., HOLMES, A. and GOLD, C. (1971) Studies on the solubility of constituents of chrysotile asbestos in vivo using radioactive tracer techniques. Env. Res. 4 558-570

MORGAN, A., DAVIES, P., WAGNER, J.C., BERRY, G. and HOLMES, A. (1977) The biological effects of magnesium leached chrysotile asbestos. Br. J. Exp. Pathol. 58 465-473

MORGAN, D.M.L., VINT, S. and RIDEOUT, J.M. (1978) Continuous flow fluorimetric assay of lysosomal enzymes. Med. Lab. Sci. 35 335-342

MORGAN, D.M.L. and ALLISON, A.C. (1980) Effects of silica and asbestos on alveolar and peritoneal macrophages: a comparative study. in Brown et al Eds. 1980, 75-81

MOSSMAN, B.T., KESSLER, J.B., LEY, B.W. and CRAIGHEAD, J.E. (1977) Interaction of crocidolite asbestos with hamster respiratory mucosa in organ culture. Lab. Invest. 36 131-139

MOSSMAN, B.T., ADLER, K.D. and CRAIGHEAD, J.E. (1980) Cytotoxic and proliferative changes in tracheal organ and cell cultures after exposure to mineral dust. in Brown et al Eds. 1980, 241-250

MULLINK, H. and von BLOMBERG, M. (1980) Influence of anti-inflammatory drugs on the interaction of lymphocytes and macrophages. Agents and Actions 10 512-515

MUNAN, L., THOUEZ, J.P., KELLY, A., GAGNE, M. and LABONTE, D. (1981) Relative leucopenia in the peripheral blood of asbestos miners; an epidemiological analysis. Scand. J. Haematol. 26 115-122

MURPHY, P.A., SIMON, P.L. and WILLOUGHBY, W.F. (1980) Endogenous pyrogens made by rabbit peritoneal exudate cells are identical with lymphocyte activating factors made by rabbit alveolar macrophages. J. Immunol. 124 2498-2501

NABARRA, B., CAVELIER, J.F., DY, M. and DIMITRIU, A. (1978) Scanning electron microscopic studies of activated macrophages in the mouse. J. Reticuloendothel. Soc. 24 489-498

NABARRA, B., DY, M., ANDRIANARISON, I. and DIMITRIU, A. (1979) Ultrastructural studies of activated mouse macrophages. J. Reticuloendothel. Soc. 25 73-83

NASEEM, S.M., TISHLER, P.V., ANDERSON, H.A. and SELIKOFF, I.J. (1978) Aryl hydrocarbon hydroxylase in asbestos workers. Am. Rev. Resp. Dis. 118 693-700

NATHAN, C.F. (1982) Secretion of oxygen intermediates: role in effector functions of activated macrophages. Fed. Proc. 41 2206-2211

NATHAN, C.F., KARNOVSKY, M.L. and DAVID, J.R. (1971) Alterations of macrophage function by mediators from lymphocytes. J. Exp. Med. 133 1356-1376

NATHAN, C.F. and ROOT, Z.A. (1977) Hydrogen peroxide release from mouse peritoneal macrophages. Dependence on sequential activation and triggering. J. Exp. Med. 146 1648-1662

NATHAN, C.F., MURRAY, H.W. and COHN, Z.A. (1980) The macrophage as an effector cell. N. Eng. J. Med. 303 622-626

NATHAN, C.F. and COHN, Z.A. (1981) Antitumor effects of hydrogen peroxide in vivo. J. Exp. Med. 154 1539-1553

NELSON, D.S. (Ed.) (1976) Immunobiology of the macrophage. Academic Press.

NELSON, D.S. (1976) Non-specific immunoregulation by macrophages and their products. in Nelson, D.S. Ed. 1976, 235-257

NELSON, D.S., SCHNEIDER, C.N. and PENROSE, J.M. (1976) Inhibition of lymphocyte transformation by products of activated macrophages and other cells. in Oppenheim, J.J. and Rosenstreich, D.L. Eds. 1976, 477-493.

NELSON, D.S., KIREMIDZIAN-SCHUMACHER, L. and STOTZKY, G. (1982) Effect of cadmium, lead and zinc on macrophage mediated cytotoxicity toward tumour cells. Env. Res. 28 154-163

NELSON, M., NELSON, D.S. and HOPPER, K.E. (1981) Inflammation and tumour growth. I Tumour growth in mice with depressed capacity to mount inflammatory responses: possible role of macrophages. Am. J. Pathol. 104 114-124

NELSON, M. and NELSON, D.S. (1981) Inflammation and tumour growth. II Tumour growth at sites of inflammation induced by mitogens in mice. Am. J. Pathol. 104 125-131

NEUGUT, A.I., EISENBERG, D., SILVERSTEIN,M., PULKBRABEK, P. and WEINSTEIN, I.B. (1978) Effect of asbestos on epithelioid cell lines. Env. Res. 17 256-265

NEWHOUSE, M.L. and THOMPSON, H. (1965) Mesothelioma of pleura and peritoneum following exposure to asbestos in the London area. Br. J. Ind. Med. 22 261-269

NEWMAN, H.A.I., SAAT, Y.A. and HART, R.W. (1980) Putative inhibitory effects of chrysotile, crocidolite and amosite mineral fibres on the more complex surface membrane glycolipids and glycoproteins. in Brown et al Eds. 1980, 147-157

NICOLSON, G.L. (1976a) Transmembrane control of the receptors on normal and tumour cells. I Cytoplasmic influence over cell surface components. Biochem. et Biophys. Acta 457 57-108

NICOLSON, G.L. (1976b) Transmembrane control of the receptors of normal and tumour cells. II Surface changes associated with transformation and malignancy. Biochem. et Biophys. Acta 458 1-72

NICOLSON, G.L. and POSTE, G. (1976) The cancer cell: dynamic aspects and modification in cell surface organization. Part I. New. Eng. J. Med. 295 197-203

NICOLSON, G.L., SMITH, J.R. and POSTE, G. (1976) Effects of local anaesthetics on cell morphology and membrane associated cytoskeletal organization in Balb/3T3 cells. J. Cell. Biol. 68 395-402

NIEDERHUBER, J.E., ALLEN, P. and MAYS, L. (1979) The expression of Ia antigen determinants on macrophages required for the in vitro antibody response. J. Immunol. 122 1342-1349

NOONAN, K.D. (1976) Role of serum in protease induced stimulation of 3T3 cell division past the monolayer stage. Nature 259 573-576

NOONAN, K.D. (1978) Lectins as probes of the transformed cell surface. in Virus Transformed Cell Membranes. Ed. Nicolau, C. Academic Press 281-371

NORTH, R.J. (1978) The concept of the activated macrophage. J. Immunol. 121 806-809

NORTH, R.J. (1981) An introduction to macrophage activation. in The Lymphokines. Vol. 3. Lymphokines and Macrophage Activation. Ed. Pick, E. 1-10

OBERDORSTER, G., BOOSE, C.H., POTT, F. and PFEIFFER, U. (1980) In vitro dissolution rates of trace elements from mineral fibres. in Brown et al Eds. 1980, 183-189

OGMUNDSDOTTIR, H. and WEIR, D.M. (1980) Mechanisms of macrophage activation. Clin. exp. Immunol. 40 223-234

OLDHAM, P.D. (1973) Asbestos in lung tissue. in Bogovski et al Eds. 1973, 231-235

OLIVER, J.M. and BERLIN, R.D. (1976) Macrophage membranes. in Nelson, D.S. Ed. 1976, 259-273

OLIVER, J.M., BERLIN, R.D., BAEHNER, R.L. and BOXER, L.A. (1977) Mechanism of microtubule disassembly in vivo: studies on normal and chronic granulomatous disease leucocytes. Br. J. Haematol. 37 311-322

OPITZ, H.G., LEMKI, H. and HEWLETT, G. (1978) Activation of T cells by a macrophage or 2 mercapto-ethanol activated serum factor is essential for induction of a primary immune response to heterologous red cells in vitro. Imm. Rev. 40 53-77

OPPENHEIM, J.J. and ROSENSTREICH, D.L. (Eds.) (1976) Mitogens in Immunobiology. Academic Press.

OTTERY, J. and GORMLEY, I.P. (1978) Some factors affecting the haemolytic activity of silicate minerals. Ann. occup. Hyg. 21 131-139

PABST, M.J. and JOHNSTON, R.B. (1980) Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide and lipopolysaccharide. J. Exp. Med. 151 101-114

PANIJEL, J. and CAYEUX, P. (1968) Immunosuppressive effects of macrophage anti-serum. Immunology 14 769-780

PAPADIMITRIOU, J.M. and WALTERS, M. N-I. (1979) Macrophage polykarya. CRC Crit. Rev. Toxicol. 6 211-255

PARKS, D.E. and WEISER, R.S. (1975) The role of phagocytosis and natural lymphokine in the fusion of alveolar macrophages to form Langhans Giant Cells. J. Reticuloendothel. Soc. 17 219-228

PASTAN, I.H. and WILLINGHAM, M.C. (1981) Journey to the center of the cell: role of the receptosome. Science 214 504-509

PEARLSTEIN, E., DIENSTMAN, S.R. and DEFENDI, V. (1978) Identification of macrophage external membrane proteins and their possible role in cell adhesion. J. Cell. Biol. 79 263-267

PELS, E. and DEN OTTER, W. (1979) Natural cytotoxic macrophages in the peritoneal cavity of mice. Br. J. Cancer 40 856-861

PENNLINE, K.J. and HERSCOWITZ, H.B. (1981) Dual role for alveolar macrophage in humoral and cell mediated immune responses: evidence for suppressor and enhancing functions. J. Reticuloendothel. Soc. 30 205-217

PERNIS, B., VIGLIANI, E.C. and SELIKOFF, I.J. (1965) Rheumatoid factor in serum of individuals exposed to asbestos. Ann. N.Y. Acad. Sci. 132 112-120

PESANTI, E.L. and NUGENT, K.M. (1981) Inhibition of macrophage phagocytosis after contact with phagocytosable particles. J. Reticuloendothel. Soc. 30 157-166

PETERS, L.J., McBRIDE, W.H., MASON, K.A. and MILAS, L. (1978) A role for T lymphocytes in tumour inhibition and enhancement caused by systemic administration of C. parvum. J. Reticuloendothel. Soc. 24 9-18

PETO, J. (1979) Dose response relationships for asbestos related disease: implications for hygiene standards. Part II. Mortality. Ann. N.Y. Acad. Sci. 330 195-204

PHAIRE-WASHINGTON, L., WANG, E. and SILVERSTEIN, S. (1980) Phorbol Myristate Acetate stimulates pinocytosis and membrane spreading in mouse peritoneal macrophages. J. Cell. Biol. 86 634-640

PICK, E. and WILNER, I. (1979) Cytoskeletal control of Concanavalin A receptor mobility in peritoneal macrophages. Exp. Cell. Res. 118 151-158

PICKRELL, J.A. (1981) Sequence of events in pulmonary injury. in Lung Connective Tissue: Location, Metabolism and Response to Injury. Ed. Pickrell, J.A. CRC Press 124-130

PIERCE, C.W. and KAPP, J.A. (1978) Function of macrophages in antibody responses in vitro. Fed. Proc. 37 86-90

PIESSENS, W.F., LACHAPELLE, F.L., LEGROS, N. and HEUSEN, J.C. (1970) Facilitation of rat mammary tumour growth by BCG. Nature 228 1210-1211

PIESSENS, W.F., CHURCHILL, W.H. and DAVID, J.R. (1975) Macrophages activated in vitro with lymphocyte mediators kill neoplastic but not normal cells. J. Immunol. 114 293-299

PITOT, H.C. (1978) Fundamentals of Oncology. Dekker 1978

PONCELOT, G., GOSSEYE, A., HODGSON, A., LINCOLN, B., WHITTAKER, E.J.W. and ZUSSMAN, J. (Eds.) (1971) The Physics and Chemistry of asbestos minerals. 2nd International Conference. Louvain, 1971. Proceedings.

POSTE, G. (1979) The tumouricidal properties of inflammatory tissue macrophages and multinucleate giant cells. Am. J. Path. 96 595-608

POSTLETHWAITE, A.E., SNYDERMAN, R. and KANG, A.H. (1976) The chemotactic attraction of human fibroblasts to a lymphocyte derived factor. J. Exp. Med. 144 1188-1203

PREGER, L. (1978) Asbestos related disease. Grune and Stratton

PUGH-HUMPHRIES, R.G.P., RICHARDSON, C.A., WILSON, A.R. and CRUIKSHANK, W.J. (1977) The effect of the macrophage cytotoxic agent iota carageenan on the growth of two transplantable murine ascites tumours. in James et al Eds. 1977, 142-153

PYLEV, L.N. and SHABAD, L.M. (1973) Some results of experimental studies in asbestos carcinogenesis. In Bogovski et al Eds. 1973, 99-105

QUIGLEY, J.P. (1979) Proteolytic enzymes of normal and malignant cells. in Surfaces of normal and malignant cells. Ed. Hynes, R.O. Wiley, 247-285

RAJAN, K.T., WAGNER, J.C. and EVANS, P.H. (1972) The response of human pleura in organ culture to asbestos. Nature 238 346-347

RAMACHANDAR, K., SCHECHTER, R., PHELPS, B. and CHAHINIAN, P. (1975) Immunocompetence in patients with pleural mesothelioma. Proc. Amer. Ass. Cancer Res. 16 142

RAZ, A., SHAHAR, A. and GOLDMAN, R. (1977) Characterization of an in vivo induced peritoneal macrophage population following intraperitoneal injection of Concanavalin A. J. Reticuloendothel. Soc. 22 445-460

REAVEN, E.P. and AXLINE, S.C. (1973) Subplasmalemmal microfilaments and microtubules in resting and phagocytizing cultivated macrophages. J. Cell. Biol. 59 12-27

REEVES, A.L., PURO, H.G. and SMITH, R.C. (1974) Inhalation carcinogenesis from various forms of asbestos. Env. Res. 8 178-202

REPORT and recommendations of the Working Group in asbestos and cancer (1965) Ann. N.Y. Acad. Sci. 132 706-721

REPORT of the Advisory Committee on asbestos cancer to the Director of the International Agency for Research on Cancer (1973) Biological Effects of Asbestos. Ann. occup. Hyg. 16 9-17

RHODES, J. (1975) Macrophage heterogeneity in receptor activity: the activation of macrophage Fc receptor function. J. Immunol. 114 976-981

RHODES, J., PLOWMAN, P., BISHOP, M. and LIPSCOMB, D. (1981) Human macrophage function in cancer: systemic and local changes detected by an assay for Fc receptor expression. J. Nat. Cancer Inst. 66 423-429

RICHARDS, R.J., WUSTEMAN, F.S. and DODGSON, K.S. (1971) The direct effects of dusts on lung fibroblasts grown in vitro. Life Sciences 10 1149-1159

RICHARDS, R.J. and MORRIS, T.G. (1973) Collagen and mucopolysaccharide production in growing lung fibroblasts exposed to chrysotile asbestos. Life Sciences 12 441-451

RICHARDS, R.J. and JACOBY, F. (1976) Light microscope studies on the effects of chrysotile asbestos and fiber glass on the morphology and reticulin formation of cultured lung fibroblasts. Env. Res. 11 112-121

RICHARDS, R.J., HEXT, P.M., DESAI, R., TETLEY, T., HUNT, J., PRESLEY, R. and DODGSON, K.S. (1977) Chrysotile asbestos: biological reaction potential. in Inhaled Particles IV. Part 2. Ed. Walton, W.H. Pergamon, 477-490

RINEHART, J.J. and BOULIVARE, T. (1977) Microfilament and microtubule function in human monocytes. J. Lab. Clin. Med. 90 737-743

ROBINSON, C., LEMEN, R. and WAGONER, J.K. (1979) Mortality patterns 1940-1975, amongst workers employed in an asbestos textile friction and packing products manufacturing facility. in Dusts and Disease. Eds. Lemen, R. and Dement, J.M. Pathotox. 131-143

ROKOSOVA, B. and BENTLEY, J.P. (1980) The effect of lysosomal enzymes in the proliferation of aortic smooth muscle cells and fibroblasts. Path. Biol. 28 493-500

ROLA-PLESZCZYNSKI, M., MASSE, S., SIROIS, P., LEMAIRE, I. and BEGIN, R. (1981) Early effects of low dose exposure to asbestos on local cellular immune responses in the lung. J. Immunol. 127 2535-2538

ROSENBERG, Y.J. (1978) Autoimmune and polyclonal B cell responses during murine malaria. Nature 274 170-172

ROSENTHAL, A.S. and SHEVACH, E.M. (1973) Function of macrophages in antigen recognition by guinea pig T lymphocytes. I Requirements for histocompatible macrophages and lymphocytes. J. Exp. Med. 138 1194-1212

ROSS, G.D. (1981) Detection of complement receptors and Fc receptors on macrophages. in Manual of Macrophage Methodology. Eds. Herscowitz, H.B., Holden, H.T., Bellanti, J.A. and Ghaffar, A. Dekker, 209-227

RUCO, L.P. and MELTZER, M.S. (1978) Macrophage activation for tumor cytotoxicity: increased lymphokine responsiveness of peritoneal macrophages during acute inflammation. J. Immunol. 120 1054-1062

RUSSELL, S.W., DOE, W.F. and McINTOSH, A.T. (1977) A noncytolytic stage of macrophage activation in Moloney sarcomas. in James et al Eds. 1977, 341-346

RYAN, G.B., UNANUE, E.R. and KARNOVSKY, M.J. (1974) Inhibition of surface capping of macromolecules by local anaesthetics and tranquilisers. Nature 250 56-57

RYAN, G.B. and MAJNO, G. (1977) Inflammation. Upjohn.

SAINT-REMY, J.M.R. and COLE, P. (1980) Interaction of chrysotile asbestos with the complement system. Immunology 41 431-437

SALMON, S.W. and HAMBURGER, A.W. (1978) Immunoproliferation and cancer: a common macrophage derived promoter substance. Lancet i 1289

SCHECHTER, G.P., WAHL, L.M. and OPPENHEIM, J.J. (1980) Suppressor monocytes in human disease: a review. in Macrophage and Lymphocytes. Nature, Function and Interactions. Part B. Eds. Escobar, M.R. and Friedman, H. Plenum 283-298

SCHMIDT, M.E. and DOUGLAS, S.D. (1977) Monocyte IgG receptor activity, dynamics and modulation: normal individuals and patients with granulomatous disease. J. Lab. Clin. Med. 89 332-340

SCHNEIDERMANN, M.A. (1974) Digestive system cancer among persons subjected to occupational inhalation of asbestos particles: a literature review with emphasis on dose response. Env. Health Perspect. 9 307-311

SCHORLEMMER, H.U., DAVIES, P. and ALLISON, A.C. (1976) Ability of activated complement components to induce lysosomal enzyme release from macrophages. Nature 261 48-49

SCHORLEMMER, H.U., HADDING, U., BITTER-SUERMANN, D. and ALLISON, A.C. (1977a) The role of complement cleavage products in killing of tumour cells by macrophages. in James et al Eds. 1977, 68-76

SCHORLEMMER, H.U., BITTER-SUERMANN, D. and ALLISON, A.C. (1977b) Complement activation by the alternative pathway and macrophage enzyme secretion in the pathogenesis of chronic inflammation. Immunology 32 929-940

SCHORLEMMER, H.U., SEDLACEK, H.H., SEILER, F.R. and BITTER-SUERMANN, D. (1981) Evidence for the participation of endogenous C3 in macrophage stimulation. in Forster and Landy, Eds. 1981, 298-300

SCHULTZ, R.M., PAPAMETHEAKIS, J.D. and CHIRIGOS, M.N. (1977) Interferon: an inducer of macrophage activation by polyanions. Science 197 674-676

SCHULTZ, R.M., PAVLIDIS, N.A., STYLOS, W.A. and CHIRIGOS, M.A. (1978) Regulation of macrophage tumoricidal function: a role for prostaglandins of the E series. Science 202 320-321

SELIKOFF, I.J., HAMMOND, E.C. and SEIDMAN, H. (1973) Cancer risk in insulation workers in the United States. in Bogovski et al Eds. 1973, 209-221

SELIKOFF, I.J. and LEE, D.H.K. (1978) Asbestos and Disease. Academic Press

SERIO,C., GANDOUR, D.M. and WALKER, W.S. (1979) Macrophage functional heterogeneity: evidence for different antibody dependent effector cell activities and expression of Fc receptors among macrophage subpopulations. J. Reticuloendothel. Soc. 25 197-206

SETHI, S., HILSCHER, W. and SCHLIPKOTER, H.W. (1971) Tissue response to intraperitoneally administered asbestos of varying types in rats. in Proc. of the IV Int. Pneumoconiosis Conference. Bucharest. Apimondia 238-240

SHANNON, B.T. and LOVE, S.H. (1980) Additional evidence for the role of hyaluronic acid in the macrophage disappearance reaction. Immunol. Commun. 9 735-746

SHARMA, S.D., PIESSENS, W.F. and MIDDLEBROOK, G. (1980) In vitro killing of tumor cells by soluble products of activated guinea pig peritoneal macrophages. Cell. Immunol. 49 379-383

SIMON, H.B. and SHEAGREN, J.N. (1972) Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. Cell. Immunol. 4 163-174

SINCOCK, A. and SEABRIGHT, M. (1975) Induction of chromosome changes in Chinese hamster cells by exposure to asbestos fibres. Nature 257 56-58

SINGER, S.J. and NICOLSON, G.L. (1972) The fluid mosaic model of the structure of cell membranes. Science 175 720-731

SIROIS, P., ROLA-PLESZCZYNSKI, M. and BEGIN, R. (1980) Phospholipase A activity and prostaglandin release from alveolar macrophages exposed to asbestos. Prostagl. Med. 5 31-37

SJOBERG, O. (1972) Effect of allogeneic cell interaction on the primary immune response in vitro. Cell types involved in suppression and stimulation of antibody synthesis. Clin. Exp. Immunol. 12 365-375

SLUITER, W., van WAARDE, D., HULSING-HESSELINK, E., ELZENGA-CLAASEN, I.L. and van FURTH, R. (1980) Humoral control of monocyte production during inflammation. in Mononuclear Phagocytes: Functional Aspects. Vol. 1. Ed. van Furth, R. Martinus Nijhoff, 325-339

SNYDERMAN, R. and FUDMAN, E.J. (1980) Demonstration of a chemotactic factor receptor on macrophages. J. Immunol. 124 2754-2757

SOLT, D.B. and FARBER, E. (1976) New principles for the analysis of chemical carcinogenesis. Nature 263 701-703

SPECTOR, W.G. (1977) Mcrophage turnover and traffic: a review. in James et al Eds. 1977, 15-18

SPRAGG, J. (1974) The plasma kinin forming system. in Mediators of Inflammation. Ed. Weissmann, G. Plenum 85-111

STAHL, P., SCHLESINGER, P.H., SIGARDSON, E., RODMAN, J.S. and LEE, Y.C. (1980) Receptor mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. Cell 19 207-215

STANTON, M.F. and WRENCH, C. (1972) Mechanism of mesothelioma induction with asbestos and fibrous glass. J. Nat. Cancer Inst. 48 797-782

STANTON, M.F., LAYARD, M., TEGERIS, A., MILLER, E., MAY, M. and KENT, E. (1977) Carcinogenicity of fibrous glass: pleural response in the rat in relation to fiber dimension. J. Nat. Cancer Inst. 58 587-597

STANTON, M.F. and LAYARD, M. (1978) The carcinogenicity of fibrous minerals. in Asbestos: Definitions and measurement methods. National Bureau of Standards Special Publication No. 506. Gaithersburg, 1977, 143-151

STEINMAN, R.M., NOGUEIRA, N., WITMER, M.D., TYDINGS, J.D. and MELLMAN, I.S. (1980) Lymphokine enhances the expression and synthesis of Ia antigens on cultured mouse peritoneal macrophages. J. Exp. Med. 152 1248-1261

STELL, P.M. and McGILL, T. (1973) Asbestos and laryngeal carcinoma. Lancet ii 416-417

STEVENS, R.H., COLE, D.A. and MEEK, E.S. (1981) Antibody dependent cell cytotoxicity in chemical induced rat pancreas and colon cancer. Env. Res. 25 372-380

STEWART, C.C., LIN, H.S. and ADLES, C. (1975) Proliferation and colony forming ability of peritoneal exudate cells in culture. J. Exp. Med. 141 1114-1132

STOSSEL, T.P. (1976) The mechanism of phagocytosis. J. Reticuloendothel. Soc. 19 237-245

STUART, A.E. (1977) The heterogeneity of macrophages: a review. in James et al Eds. 1977, 1-14

SYKES, S.E., MORGAN, A. and HOLMES, A. (1980) The haemolytic activity of chrysotile asbestos. in Brown et al Eds. 1980, 113-119

TAFFET, S.M. and RUSSELL, S.W. (1980) Macrophage mediated tumour cell killing: regulation of expression of cytolytic activity by Prostaglandin E. J. Immunol. 126 424-427

TANAKA, A., NAGAO, S., IMAI, K. and MORI, R. (1980) Macrophage activation by muramyl dipeptide as measured by macrophage spreading and attachment. Microbiol. Immunol. 24 547-557

TEJWANI, C.P., FERTEL, R., HART, R.W. and ALLISON, D.K. (1980) Effect of asbestos and chemical carcinogens on the cyclic nucleotide system of human fibroblasts. J. Env. Path. Tox. 4 67-70

TEMPLE, A. and ALLISON, A.C. (1980) Cytolysis of fibroblasts by C3a. Br. J. Cancer 42 21-25

TENU, J.P., LEDERER, E. and PETIT, J.F. (1980) Stimulation of thymocyte mitogenic protein secretion and of cytostatic activity of mouse peritoneal macrophages by trehalose dimycolate and muramyl dipeptide Eur. J. Immunol. 10 647-653

THIOLLET, J., JAURAND, M.C., KAPLAN, H., BIGNON, J. and HOLLANDE, E. (1978) Culture procedure of mesothelial cells from the rat parietal pleura. Biomedicine 29 69-73

THOMSON, R., KILROE-SMITH, T.A. and WEBSTER, I. (1978) The effects of asbestos associated metal ions on the binding of benzo(∝) pyrene to macromolecules in vitro. Env. Res. 15 309-319

TIMBRELL, V. (1969) Characteristics of the International Union against cancer standard reference samples of asbestos. in Pneumoconiosis: Proceedings of the International Conference. Johannesburg. Ed. Shapiro, H.A. Oxford Univ. Press 28-36

TIMBRELL, V. (1973) Progress in Physics and Chemistry. in Bogovski et al Eds. 1973, 13-16

TIMBRELL, V. (1980) Uses of the UICC samples. in Biological Effects of Mineral Fibres. Ed. Wagner, J.C. IARC Pub. No. 30 127-142

TIMBRELL, V. and SKIDMORE, J.W. (1968) Significance of fibre length in experimental asbestosis. Biologische Wirkungen des Asbestos Internationale. Konferenz Dresden Deutsches Zentral Institut fur Arbeitsmedizin. 52-56

TIMBRELL, V., GILSON, J.C. and WEBSTER, I. (1968) UICC standard reference samples of asbestos. Int. J. Cancer 3 406-408

TOIVANEN, A., SALMIVALLI, M. and MOLNAR, G. (1976) Pulmonary asbestosis and autoimmunity. Br. Med. J. i 691-692

TOPPING, D.C. and NETTESHEIM, P. (1980) Two stage carcinogenesis studies with asbestos in Fischer 344 rats. J.Nat. Cancer Inst. 65 627-630

TSAN, M.F. and BERLIN, R.D. (1971) Effect of phagocytosis on membrane transport of non-electrolytes. J. Exp. Med. 134 1016-1035

TURNER-WARWICK, M. and PARKES, W.R. (1970) Circulating rheumatoid and antinuclear factors in asbestos workers. Br. Med. J. iii 492-495

TURNER-WARWICK, M. and HASLAM, P. (1971) Autoantibodies in some chronic fibrosing lung diseases. I Non organ specific autoantibodies. Clin. Allerg. 1 83-95

UICC WORKING GROUP (1965) Report and recommendations of the working group on asbestos and cancer. Ann. N.Y. Acad. Sci. 132 706-721

UNANUE, E.R. (1968) Properties and some uses of anti-macrophage antibodies. Nature 218 36-38

UNANUE, E.R. (1978a) The regulation of lymphocyte functions by the macrophage. Immunol. Rev. 40 227-255

UNANUE, E.R. (1978b) Macrophage functions in immunity: Introduction to symposium. Fed. Proc. 37 77-78

UNANUE, E.R., KIELY, J-M. and CALDERON, J. (1976) The modulation of lymphocyte functions by molecules secreted by macrophages. II Conditions leading to increased secretion. J. Exp. Med. 144 155-166

UNANUE, E.R. and ROSENTHAL, A.S. (Eds.) (1980) Macrophage Regulation of Immunity. Academic Press

VALERIO, F., VEGGI, M. and SANTI, L. (1980) Adsorption isotherms of albumin and ferritin on Rhodesian chrysotile. Env. Res. 21 186-189

van FURTH, R. (1980) Cells of the mononuclear phagocyte system: nomenclature in terms of sites and conditions. in Mononuclear Phagocytes. Functional aspects. Part I. Ed. van Furth, R. Martinus Nijhoff 1-30

van FURTH, R. (1981) Development of mononuclear phagocytes. in Forster and Landy, Eds. 1981, 3-7

van FURTH, R., COHN, Z.A., HIRSCH, J.G., HUMPHREY, J.H., SPECTOR, W.G. and LANGEVOORT, H.L. (1972) The mononuclear phagocyte system: a new classification of macrophages, monocytes and their pre-cursor cells. Bull. World Health Org. 46 845-852

van FURTH, R., DIESSELHOFF den DULK, M.M.C., RAEBURN, J.A., van ZWET, T.L., CROFTON, R. and BLUSSE van OUD ALBLAS, A. (1980) Characteristics, origin and kinetics of human and murine mononuclear phagocytes. in Mononuclear phagocytes: Functional aspects. Vol. 1. Ed. van Furth, R. Martinus Ninhoff 279-298

van WAARDE, D., HULSING HESSELINK, E. and van FURTH, R. (1978) Humoral control of monocytopoesis by an activator and an inhibitor. Agents and Actions 8 432-437

VARESIO, L. and HOLDEN, H.T. (1980) Suppression of lymphokine production. I Macrophage mediated inhibition of MIF production. Cell. Immunol. 56 16-28

VARESIO, L., HOLDEN, H.T. and TARAMELLI, D. (1981) Suppression of lymphokine production. II Macrophage dependent inhibition of production of macrophage activating factor. Cell. Immunol. 63 279-292

VASSALLI, J.D., HAMILTON, J. and REICH, E. (1977) Macrophage plasminogen activator: induction by Concanavalin A and Phorbol Myristate Acetate. Cell 11 695-705

VORWALD, A.J., DURKAN, T.M. and PRATT, P.C. (1951) Experimental studies of asbestosis. Arch. Ind. Hyg. 3 1-43

WADE, M.J., LIPKIN, L.E., STANTON, M.F. and FRANK, A.L. (1980) P388 D1 in vitro cytotoxicity assays as applied to asbestos and other minerals: its possible relevance to carcinogenicity. in Brown et al Eds. 1980, 351-357

WADEE, A.A. and RABSON, A.R. (1981) Production of a suppressor factor by adherent cells from Mycobacterium tuberculosis infected guinea pigs. Clin. exp. Immunol. 45 427-432

WAGNER, J.C. (1962) Experimental production of mesothelial tumours of the pleura by the implantation of dust in laboratory animals. Nature 196 180-181

WAGNER, J.C. (1963) Asbestosis in experimental animals. Brit. J. Industr. Med. 20 1-12

WAGNER, J.C., SLEGGS, C.A. and MARCHAND, P. (1960) Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. Brit. J. Industr. Med. 17 260-271

WAGNER, J.C. and BERRY, G. (1969) Mesothelioma in rats following inoculation with asbestos. Br. J. Cancer 23 567-587

WAGNER, J.C., BERRY, G. and TIMBRELL, V. (1970) Mesotheliomas in rats following intra-pleural inoculation of asbestos. in Pneumoconiosis. Proc. Int. Conf. Johannesburg 1969. Ed. Shapiro, H.E. Oxford Univ. Press 216-219

WAGNER, J.C., BERRY, G., SKIDMORE, J.W. and TIMBRELL, V. (1974) The effects of inhalation of asbestos in rats. Br. J. Cancer 29 252-269

WAGNER, J.C., HILL, R.J., BERRY, G. and WAGNER, M.M.F. (1980) Treatments affecting the rate of asbestos induced mesotheliomas. Br. J. Cancer 41 918-922

WAGNER, M.M.F. (1979) Thymectomy and asbestos induced mesotheliomas in rats. Br. J. Cancer 39 337-341

WAGNER, M.M.F., WAGNER, J.C., CAMPBELL, M.J. and EDWARDS, R.E. (1979) Sequential immunological studies on an asbestos exposed population I Factors affecting peripheral blood leucocytes and T lymphocytes. Clin. exp. Immunol. 38 323-331

WAHL, L.M., WAHL, S.M. and McCARTHY, J.B. (1980a) Adjuvant activation of macrophage functions. in Macrophage regulation of immunity. Eds. Unanue, E.R. and Rosenthal, A.S. Academic Press, 491-504

WAHL, S.M., WAHL, L.M. and MERGENHAGEN, S.E. (1980b) Lymphokine and monokine regulation of fibroblast function. in Biochemical Characterization of Lymphokines. Eds. de Wec, A.L., Kristensen, F. and Landy, M. Academic Press, 267-272

WALDREP, J.C. and REESE, A.C. (1981) Polyclonal immunoglobulin synthesis induced by a macrophage factor acting via T cells. Immunology 42 619-628

WALDREP, J.C., MOHANAKUMAR, T. and KAPLAN, A.M. (1981) Characterization of human monocyte macrophage differentiation antigens. in Forster and Landy, Eds. 1981, 79-81

WALKER, W.S. (1974) Functional heterogeneity of macrophages: subclasses of peritoneal macrophages with different antigen binding activities and immune complex receptors. Immunology 26 1025-1037

WALKER, W.S. (1976) Functional heterogeneity of macrophages. in Nelson, D.S. Ed. 1976, 91-110

WALTERS, M. N-I. and PAPADIMITRIOU, J.M. (1978) Phagocytosis: a review. CRC Crit. Rev. Toxicol. 5 377-421

WARD, M., FRANK, A.L., WENK, M., DEVOR, D. and TARONE, R.E. (1980) Ingested asbestos and intestinal carcinogenesis in F344 rats. J. Env. Path. Tox. 3 301-312

WARNER, L.A., HOLT, P.G. and MAYRHOFER, G. (1981) Alveolar macrophages. VI Regulation of alveolar macrophage mediated suppression of lymphocyte proliferation by a putative T cell. Immunology 42 137-147

WEHNER, A.P., BUSCH, R.H., OLSON, R.J. and CRAIG, D.K. (1975) Chronic inhalation of asbestos and cigarette smoke by hamsters. Env. Res. 10 368-383

WEIR, D.M. and OGMUNDSDOTTIR, H.M. (1977) Non-specific recognition mechanisms by mononuclear phagocytes. Clin. exp. Immunol. 30 323-329

WEIR, D.M. and OGMUNDSDOTTIR, H.M. (1980) Cellular recognition by phagocytes: role of lectin like receptors. in Mononuclear Phagocytes. Functional Aspects. Part I. Ed. van Furth, R. Martinus Nijhoff, 865-881

WEITZMAN, S.A. and STOSSEL, T.P. (1981) Mutation caused by human phagocytes. Science 212 546-547

WERB, Z. and COHN, Z. (1972) Plasma membrane synthesis in the macrophage following phagocytosis of polystyrene lates particles. J. Biol. Chem. 247 2439-2446

WERB, Z. and CHIN, J. (1981) The secretion phenotypes of macrophage populations. in Forster and Landy, Ed. 1981, 301-303

WHITAKER, D. and SHILKIN, K.B. (1981) Carcinoembryonic antigen in tissue diagnosis of malignant mesothelioma. Lancet i 1369

WHITTAKER, E.J.W., CRESSEY, B.A. and HUTCHISON, J.L. (1982) Sidestepping of multiple chain lamellae in grunerite asbestos. Min. Mag. 46 273-274

WHITWELL, F. and RAWCLIFFE, R.M. (1971) Diffuse malignant pleural mesothelioma and asbestos exposure. Thorax 26 6-22

WHITWELL, F., NEWHOUSE, M.L. and BENNET, D.R. (1974) A study of the histological cell types of lung cancer in workers suffering from asbestosis in the United Kingdom. Br. J. Industr. Med. 31 298-303

WILLIAMS, D.A., BOXER, L.A., OLIVER, J.M. and BAEHNER, R.L. (1977) Cytoskeletal regulation of Concanavalin A capping in pulmonary alveolar macrophages. Nature 267 255-257

WILLIAMS, D.A., LESSARD, J., BOXER, L.A., REIN, D. and BAEHNER, R.L. (1979) Metabolic and structural requirements for Concanavalin A capping in phagocytic cells. Proc. Soc. Exp. Biol. Med. 162 34-37

WILSON, M.R., GAUMER, H.R. and SALVAGGIO, J.E. (1977) Activation of the alternative complement pathway and generation of chemotactic factors by asbestos. J. Allergy Clin. Immunol. 60 218-222

WING, E.J. and REMINGTON, J.S. (1977) Studies on the regulation of lymphocyte reactivity by normal and activated macrophages. Cell. Immunol. 30 108-121

WING, E.J. and REMINGTON, J.S. (1980) Activated macrophages pre-incubated in vitro enhance rather than suppress mitogen stimulated lymphocyte transformation. Immunology 180 239-246

WIRTH, T. (1975) The effect of asbestos cement, UICC asbestos samples and quartz in the peritoneum of the mouse (German). Pathol. Microbiol. 42 15-28

WOODRUFF, M.F.A., INCHLEY, M.P. and DUNBAR, N. (1972) Further observations on the effect of C. parvum and anti-tumour globulin on syngeneically transplanted tumours. Br. J. Cancer 26 67-76

WOODWARD, J.G., FERNANDEZ, P.A. and DAYNES, R.A. (1979) Cell mediated immune response to syngeneic or induced tumours. III Requirement for an Ia^+ macrophage in the in vitro differentiation of cytotoxic T lymphocytes. J. Immunol. 122 1196-1202

WRIGHT, G. (1969) Asbestos and health in 1969. Am. Rev. Resp. Dis. 100 467-479

WRIGHT, G.W. and KUSCHNER, M. (1977) The influence of varying lengths of glass and asbestos fibres on tissue response in guinea pigs. in Inhaled Particles IV, Part 2. Ed. Walton, W.H. 455-472

YIN, H.L., ALEY, S., BIANCO, C. and COHN, Z. (1980) Plasma membrane polypeptides of resident and activated mouse peritoneal macrophages. Proc. N.Y. Acad. Sci. 77 2188-2191

ZINKERNAGEL, R.M. and BLANDEN, R. (1975) Macrophage activation in mice lacking thymus derived (T) cells. Experientia 31 591-593

ZUCKERMAN, S.H. and DOUGLAS, S.D. (1979) Dynamics of the macrophage plasma membrane. Ann. Rev. Microbiol. 33 267-307

ZURIER, R.B. (1974) Prostaglandins. in Mediators of Inflammation. Ed. Weissmann, G. Plenum 163-180

ZUSSMAN, J. (1978) The crystal structure of amphibole and serpentine minerals. in Proceedings of Workshop on Asbestos: definitions and measurements. Maryland, 1977. Eds. Gravatt, C.C., La Fleur, P.D., and Heinreich, F.J. 1978, 36-48

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Concanavalin A receptors and capping in control and activated macrophages

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Summary

Macrophages activated *in vivo* with *Corynebacterium parvum* and asbestos showed an increased sub-population of cells which capped spontaneously on incubation with fluoresceinated Concanavalin A compared to saline-induced control macrophages. This capping was unaffected by colchicine but was inhibited by cytochalasin B. The spontaneous capping of activated macrophages did not appear to be directly related to the total number of Concanavalin A receptors as measured by specific ^{125}I-labelled Concanavalin A binding to activated and control macrophages. Capping also did not appear to be related to the position of Concanavalin A receptors as judged by trypsin accessibility of bound ^{125}I-labelled Concanavalin A.

Introduction

Macrophages occupy a central role in the inflammatory (Allison *et al.*, 1978) and immune systems (see papers in Unanue & Rosenthal, 1980). The plasma membrane of the macrophage is seen as the primary site of the sensory and effector roles of this cell including endocytosis (Walters & Papadimitriou, 1978), macrophage–lymphocyte interactions in antigen recognition (Rosenthal *et al.*, 1975; Lipscomb *et al.*, 1981), handling of immune complexes via specific receptors for immunoglobulin and complement (Mantovani, 1981) and functional responses to lymphocyte products via specific lymphokine receptors (David *et al.*, 1980).

Macrophages can undergo profound changes in their metabolic, cytochemical, morphological and secretory status in response to a variety of agents (Ogmundsdottir & Weir, 1980) and, while there is some disagreement over terminology, these changes can be collectively called activation (Cohn, 1978; Morahan, 1980). Macrophage activation results in changes in many surface properties such as ectoenzyme activity, pinocytosis (Cohn, 1978), Fc receptor activity (Rhodes, 1975) and surface morphology (Nabarra *et al.*, 1978).

Macrophages can specifically bind a number of lectins to their plasma membrane including Concanavalin A (Oliver & Berlin, 1975), *Lotus tetragonolobus* lectin (Homma *et al.*, 1981), wheat germ agglutinin (Hadley *et al.*, 1977), phytohaemagglutinin and pokeweed mitogen (Loor & Roelants, 1974). Concanavalin A (Con A) specifically binds to D-mannosyl and D-glucosyl residues at the cell surface and the macrophage Con A receptor has been studied with regard to the effect of spreading and phagocytosis (Lutton, 1973), ligand-induced capping (Williams *et al.*, 1979) and transmembrane organization (Adachi *et al.*, 1980).

Intraperitoneal asbestos injection in mice induces an activated macrophage population (Donaldson *et al.*, 1982) and activated macrophages may play a role in the aetiology of asbestos-related disease (Miller, 1978); *Corynebacterium parvum*-activated macrophages are well known for their growth regulating effect on tumour cells (see papers in James *et al.*, 1977).

In the present paper, we report on the mobility and number of Con A receptors on macrophages activated *in vivo* with asbestos and C. *parvum*. These experiments form part of a wider study into the effects of asbestos on macrophage membranes.

Materials and methods

Mice

Male CBA/Ca mice 12–16 weeks old at the time of injection, were used throughout. Mice were housed under conventional conditions and were killed by ether inhalation when required.

Macrophages

Control macrophages were obtained three days after intraperitoneal injection of 0.5 ml sterile Dulbecco's phosphate-buffered saline 'A' (Dul A). Activated macrophages were obtained five days after the intraperitoneal injection of 0.2 ml (1.4 mg) of *Corynebacterium parvum* (*C. parvum*) (heat killed; Wellcome) or three days after intraperitoneal injection of 2.5 mg chrysotile asbestos (UICC sample) in 0.5 ml sterile Dul A. In some experiments macrophages were obtained three days after intraperitoneal injection of 1% latex spheres (Bacto latex 8.1 μm diameter; Difco) in Dul A or 10% Proteose peptone (Difco) in Dul A.

Macrophage cultures were prepared by washing out the peritoneal cavity with 3 × 2 ml of RPMI 1640 medium (Gibco) containing 10 U/ml heparin (Pularin). In ^{125}I-labelled Con A binding experiments, the proportion of adherent cells was ascertained and found to be 74.2% for saline, 49.3% for chrysotile and 65.6% for C. *parvum*. Activated peritoneal exudate cells were adjusted to yield the same concentration of adherent cells as saline peritoneal exudate cells – 4.5 × 10^6/ml in RPMI 1640 plus antibiotics and 10% foetal calf serum (Gibco). One hundred microlitres of exudate cell suspension were pipetted onto washed 6 × 22 mm glass coverslips and incubated for 1 h at 37° C. These coverslips were then vigorously washed in phosphate-buffered saline (PBS) to remove non-adherent cells; the resulting adherent cell cultures are referred to as macrophage cultures.

Concanavalin A

Con A was obtained from Pharmacia (London), fluoresceinated Con A (FITC-Con A) from Sigma (London), and Na^{125}I from Amersham International (Amersham).

FITC-Con A-induced capping

Two hundred microlitres of FITC-Con A (20 μg/ml Dul A) were pipetted onto macrophage cultures which were then incubated for 20 min at 37° C. Coverslip cultures were washed, fixed in ethanol and mounted on slides in PBS–glycerol (1:1 v/v). Coverslips were examined in a fluorescence microscope and the percentage of capped macrophages in 200 cells was assessed for each coverslip.

In experiments on pharmacological modification of capping, colchicine (0.1 mM) and cytochalasin B (0.1 mM) (Sigma, London) were prepared in PBS and 100 μl of each drug added to macrophage cultures; cultures were then incubated at 37° C for up to 1 h prior to treatment with FITC-Con A at 37° C for 20 min.

The specificity of the FITC-Con A binding was demonstrated by a reduction to a barely detectable fluorescence by pre-incubation either with 20 μg/ml unlabelled Con A or in the presence of 50 mM methyl mannoside, the specific ligand for Con A.

^{125}Iodination of Con A

Con A was iodinated by the chloramine T method of Hunter (1973) and passed down a Sephadex G25 column to remove unbound ^{125}I. In the pooled ^{125}I-labelled Con A-containing fractions eluted from the G25 column, 90–95% of ^{125}I counts were trichloracetic acid-precipitable and the specific activity was, on average, 90 μCi/mg Con A.

^{125}I-labelled Concanavalin A binding assay

One hundred microlitres of ^{125}I-labelled Con A (200 μg/ml) were pipetted onto macrophage coverslip cultures, or coverslip only controls for non-specific binding, and left for 15 min at room temperature. Coverslips were then extensively washed in PBS and the coverslip associated counts determined in a gamma counter. The average background counts due to non-specific binding to coverslips ranged from 10–20% of the binding to macrophage coverslip cultures. A minimum of eight replicate coverslip cultures was used for each condition.

The specificity of the ^{125}I-labelled Con A binding to macrophages was demonstrated by a reduction of the background binding in the presence of 50 mM methyl mannoside, the specific ligand for Con A. Any confusing effects due to endocytic uptake of ^{125}I-labelled Con A was ruled out by demonstrating no effect on binding following pre-treatment with the endocytosis inhibitors iodoacetate (1 mM) and sodium azide (2 mM).

For phagocytosis experiments, 100 μl 1% Latex spheres (Bacto latex 0.81 μm diameter; Difco) were added to macrophage cultures. Cultures were then incubated for 1 h at 37° C followed by vigorous and extensive washing to remove unphagocytosed Latex before assaying for ^{125}I-labelled Con A binding.

Trypsin accessibility of Con A binding sites

Following binding of ^{125}I-labelled Con A, coverslips were placed in 0.1 mg/ml trypsin (Difco) in PBS and allowed to incubate for 15 min at room temperature. Coverslips were then agitated in the trypsin solution and removed with forceps; trypsin-associated and coverslip-associated counts were then determined. The trypsin-released counts were expressed as a percentage of the total counts.

Statistics

The significance of differences was analysed using Student's *t*-test.

Results

FITC-Con A-induced capping

Saline-induced control macrophages had a small population of cells which formed caps spontaneously on incubation with FITC-Con A at 37° C (Table 1). Both C. *parvum*-and asbestos-activated macrophages had populations of spontaneously capping macrophages approximately eight times greater than the saline controls (Table 1). Both Latex- and Proteose peptone-induced macrophages had proportions of spontaneously capping macrophages similar to the saline-induced macrophages (Table 1).

Time course of capping

The time course of FITC-Con A-induced capping was similar for both C. *parvum*- and asbestos-activated macrophages (Fig. 1) reaching a plateau by 20 min and increasing little thereafter.

Table 1. Proportion of macrophages from various sources capping on incubation with FITC-Con A (20 μg/ml) at 37° C or room temperature (20° C). 200 cells counted.

Macrophage source	*Conditions of FITC-Con A treatment*	*Mean % (± S.D.) capped macrophages*	*Number of experiments*
3 day saline	20 min, 37° C	4.1 ± 2.3	8
3 day asbestos	20 min, 37° C	32.2 ± 4.2	5
5 day C. *parvum*	20 min, 37° C	32.4 ± 5.0	6
5 day C. *parvum*	20 min, room temp.	1.8 ± 0.8	3
3 day Proteose peptone	20 min, 37° C	1.2 ± 1.1	2
3 day Latex	20 min, 37° C	3.2 ± 0.9	1

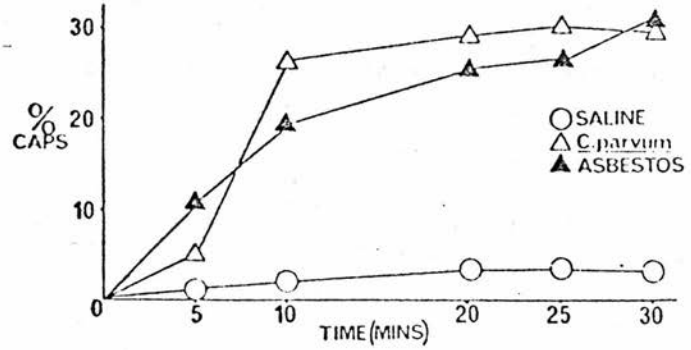

Fig. 1. Time course of Con A-induced capping in asbestos- and C. *parvum*-activated macrophages and in saline control macrophages incubated with FITC-Con A (20 μg/ml) at 37° C.

Pharmacological modification of capping

Colchicine and cytochalasin pre-treatment did not significantly affect the incidence of capping in control macrophages. Colchicine pre-treatment caused a slight insignificant increase in the proportion of capping activated macrophages whereas cytochalasin pre-treatment reduced capping of activated macrophages to control levels (Table 2).

Table 2. Effect of pre-treatment with the microtubule-disrupting drug colchicine, and the microfilament-disrupting drug cytochalasin B, on FITC-Con A-induced capping in control and activated macrophages. 200 cells counted.

Macrophage source	*Pre-treatment before FITC-Con A* (20 min., 37° C)	*Mean % (± S.D.) capped macrophages*	*Number of experiments*
3 day saline	Saline 1 h, 37° C	4.2 ± 1.6	3
	0.1 mM colchicine (1 h, 37° C)	5.6 ± 4.8	4
	0.1 mM cytochalasin 'B' (1 h, 37° C)	3.0 ± 5.2	4
3 day asbestos	Saline 1 h, 37° C	32.7 ± 4.1	3
	0.1 mM colchicine (1 h, 37° C)	39.5 ± 10.5	3
	0.1 mM cytochalasin 'B' (1 h, 37° C)	6.0 ± 2.9	3

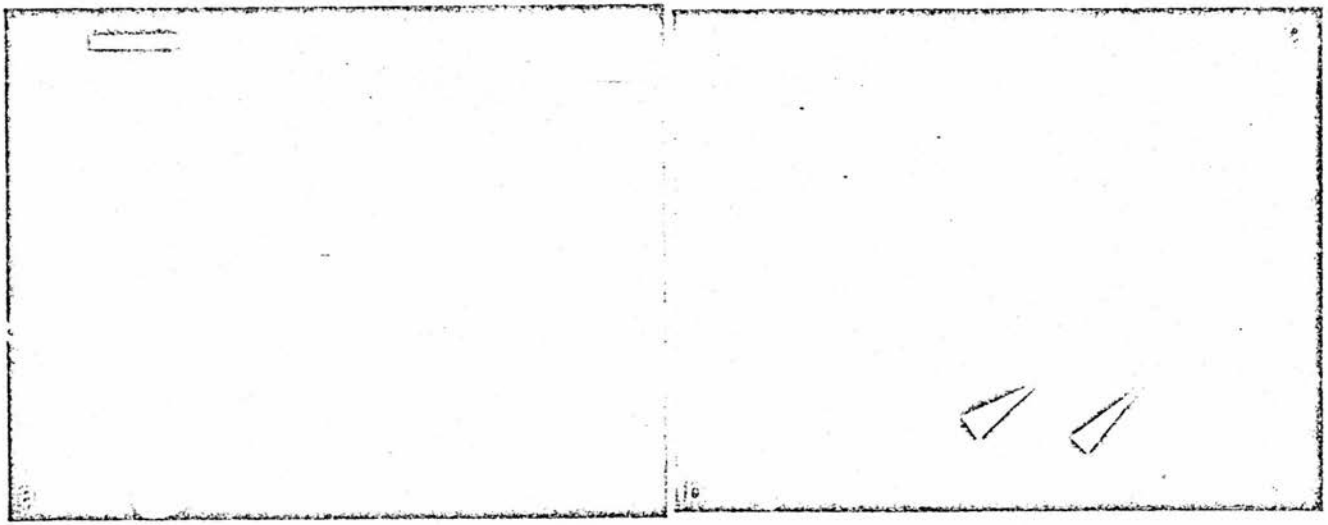

Fig. 2. (a) Three day asbestos-induced macrophages incubated with FITC-Con A (20 μg/ml) at 37° C for 1 h; capping cells arrowed. (b) Three day saline-induced macrophages treated as above; no capping sub-population. Scale bar: 10 μm.

Table 3. (a) Effect of Latex phagocytosis on ^{125}I-labelled Concanavalin A binding; non-specific binding to control Con A to equal numbers of control and activated macrophages; non-specific binding to control coverslips = 10 957 ± 367 counts per minute

Macrophage source	[^{125}I] *Counts/coverslip* (mean ± S.D.)
(a)	
Control macrophages*	28 046 ± 3945
Control macrophages after Latex phagocytosis	16 227 ± 2515
(b)	
Control macrophages	38 960 ± 2616
3 day asbestos	36 361 ± 2084
5 day *C. parvum*	52 207 ± 4855

*Control macrophages = 3 day saline induced.

Appearance of caps

The caps produced in activated macrophages following incubation with FITC-Con A for 20 min at 37° C are shown in Fig. 2 along with saline-induced non-capping macrophages for comparison. Some evidence for pinocytic uptake of FITC-Con A was evident as pale speckled fluorescence, most noticeably in the activated populations.

Binding of ^{125}I-labelled Con A; effect of phagocytosis

Phagocytosis of Latex particles by control macrophages resulted in a significant (42.4%; $P < 0.001$) reduction in ^{125}I-labelled Con A binding compared to appropriate controls (Table 3).

Binding of ^{125}I-labelled Con A to activated macrophages

^{125}I-labelled Con A bound to asbestos-activated macrophages to the same extent as to control macrophages (Table 3); *C. parvum*-activated macrophages bound significantly more ^{125}I-labelled Con A (27.2%; $P < 0.001$) than control macrophages.

Table 4. Percentage of ^{125}I-labelled Con A bound to control and activated macrophages released by trypsin (0.1 mg/ml, 15 min, room temp.).

Macrophage source	*Mean % (± S.D.) trypsin-released counts*
3 day saline	31.9 ± 2.9
3 day asbestos	33.2 ± 1.3
5 day *C. parvum*	30.7 ± 2.4

Trypsin accessibility of ^{125}I-labelled Con A binding sites

The proportion of ^{125}I-labelled Con A binding sites which were accessible to trypsinization was the same for control and both types of activated macrophage (Table 4).

Discussion

Experiments to determine the proportion of macrophages which formed caps on incubation with FITC-Con A revealed that both C. *parvum*- and asbestos-activated macrophages had capping sub-populations of 32%. This figure contrasts with 4% capping macrophages in the control, saline-induced macrophages. Since Latex-induced macrophages had control levels of capping, it is clear that phagocytosis alone is not always a sufficient stimulus for inducing the capping sub-population. Since Proteose peptone-induced macrophages also had low levels of spontaneously Con A capping cells, not all macrophage-activating agents induce an increased capping sub-population.

Evidence that macrophage activation may play a role in Con A-induced capping exists in previously published work. Thus Pick & Wilner (1979) found that resting peritoneal macrophages had no capping cells while Williams *et al.* (1979) using peritoneal macrophages induced with sodium caseinate, a procedure likely to induce some activation, found approximately 10% spontaneously capping macrophages. Similarly, the finding of approximately 30% spontaneously Con A capping guinea-pig alveolar macrophages is in keeping with the suggestion that resting alveolar macrophages, as measured by several parameters, are in a consistently higher activational state than resting peritoneal macrophages (Hopper *et al.*, 1979).

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Pharmacological modification of capping showed that, in our hands, colchicine had virtually no effect in promoting capping although previous studies have demonstrated that with colchicine treatment the level of spontaneously induced Con A caps in macrophages can be increased 2–30-fold (Williams *et al.*, 1977, 1979; Pick & Wilner, 1979). It is clear, however, that in all these studies, the majority of macrophages were not colchicine-sensitive, being either spontaneously capping or non capping despite colchicine treatment. In our assay system with CBA mouse peritoneal macrophages, the mobility of Con A receptors is clearly not inhibited by colchicine-sensitive microtubules. The abolition of capping in activated macrophages following cytochalasin B pre-treatment confirms that microfilaments are necessary for Con A-induced capping in peritoneal macrophages (Williams *et al.*, 1979) and spleen cells, presumed to be lymphocytes (De Petris, 1974).

Nathan & Root (1977) have reported that macrophages activated by a range of agents *in vivo* responded to Con A and other membrane perturbing agents by releasing hydrogen peroxide the important endogenous macrophage microbicidal agent. Oliver *et al.* (1977), from their study, suggested that an increase in hydrogen peroxide content in polymorphonuclear cells at least, could have a direct role in tubulin oxidation and therefore inhibition of microtubule assembly, leading to an increase in capping. This explanation of capping in activated macrophages, reflecting an increase in oxidative

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metabolism, is an attractive one despite our failure to find an obvious role for microtubules using colchicine and the reported difference in the cytoskeletal requirement for capping between macrophages and polymorphonuclear leucocytes (Williams *et al.*, 1979).

In order to ascertain whether the increased ability of activated macrophages to cap spontaneously on incubation with FITC-Con A was related to the total number of Con A receptors on the surface of control and activated macrophages, the binding of ^{125}I-labelled Con A to such macrophage populations was compared. In initial experiments, the finding of a significant reduction in ^{125}I-labelled Con A binding following Latex phagocytosis was in agreement with the original experiments of Lutton (1973); this suggested that our assay system, which was based on Lutton's, was comparable.

The total ^{125}I-labelled Con A binding to asbestos-activated macrophages was the same as for control macrophages. Previous work concerning the effect of environmental pollutants on lectin binding to alveolar macrophages has shown that ozone and nitrogen dioxide do not cause an increase in the binding of ^{3}H-labelled Con A to alveolar macrophages compared to control macrophages (Goldstein *et al.*, 1977). Hadley *et al.* (1977), however, found increased binding of the lectin wheatgerm agglutinin to ozone-treated macrophages.

C. parvum-activated macrophages bound approximately 30% more ^{125}I-labelled Con A than control and asbestos-activated macrophages. Since activation can occur via several different pathways or combinations of pathways (Rabinowitz & Hamburg, 1980) and since activation is not an all or none process but is rather a sequential adoption of properties obtained through differentiation signals, it is not surprising that there is considerable variation in activational phenotypes (Cohn, 1978; Hopper *et al.*, 1979). Due to the very different natures of asbestos and *C. parvum*, it is likely that they activate macrophages via different pathways. Asbestos- and C. parvum-activated macrophages possess different activational phenotypes, *C. parvum* macrophages being tumouricidal while asbestos-activated macrophages are not (Donaldson *et al.*, 1982). The increased binding of ^{125}I-labelled Con A to *C. parvum*-activated macrophages could, therefore, reflect a functionally related difference in the membrane of the tumouricidally activated macrophage. Increased binding could also be due to increased cell surface polysaccharide as reported for macrophages involved in cell-mediated immunity (Hammond & Dvorak, 1972).

The foregoing discussion, on the heterogeneity of expression of the activated macrophage phenotype, emphasizes the danger of regarding the Con A capping described above as a universal member of macrophage activation until it has been shown to occur with a range of activating agents *in vivo* and *in vitro*.

While trypsin accessibility of Con A binding sites is reported to be altered between fibroblasts and their transformed counterparts (Marques, 1978), the changes from the unactivated to the activated state in peritoneal macrophages did not result in a change in the proportion of membrane-bound ^{125}I-labelled Con A which was trypsin releasable.

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Thus, although the total number of Con A binding sites was increased in the *C. parvum*-induced macrophages compared to both saline- and asbestos-induced macrophages, there was no difference in the trypsin accessibility of these additional binding sites.

Conclusion

These experiments have demonstrated that interaction between the macrophage membrane Con A receptor glycoprotein and the cytoskeleton is altered in a sub-population comprising approximately one-third of activated macrophages, as shown by the ability of this sub-population to cap spontaneously on incubation with FITC-Con A. The capping was found to be microfilament-mediated. The ability to cap in the activated macrophage sub-population did not appear to be due simply to an alteration in the number of Con A receptor sites on activation, since two different activating agents were used to induce capping but only one caused a significant increase in Con A receptor sites. Trypsin treatment released similar amounts of bound Con A from the surfaces of control and activated macrophages suggesting that, by the criterion of trypsin accessibility, the position of the Con A receptor in the complex milieu of the macrophage surface was the same in both control and activated macrophages. These results support previous findings that the macrophage Con A receptor glycoprotein is a transmembrane molecule and can be linked to the cytoskeleton as shown by its ability to cap under various conditions. We suggest that macrophage activation may be a factor in the contradictory reports on the proportion of peritoneal macrophages which can cap spontaneously with Con A treatment; differences in activation could also explain the difference in spontaneous Con A capping reported between alveolar and peritoneal macrophages. Such an alteration in membrane glycoprotein–cytoskeletal association can be envisaged as a further manifestation of the well-documented membrane related changes which accompany macrophage activation and could also reflect their increased oxidative metabolism.

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References

ADACHI, I., KATAGIRI, T., TERAO, T. & OSAWA, T. (1980) Concanavalin A receptor proteins of plasma membranes of guinea pig macrophages. *Cell Struct. Funct.* 5, 247–57.

ALLISON, A. C., FERLUGA, J., PRYDZ, H. & SCHORLEMMER, H. U. (1978) The role of macrophage activation in chronic inflammation. *Agents Actions* 8, 27–34.

COHN, Z. A. (1978) The activation of mononuclear phagocytes. Fact, fancy and future. *J. Immun.* **121**, 813–6.

DAVID, J. R., REMOLD, H. G., HIGGINS, T. J., LIU, D. Y. & CHURCHILL, W. H. (1980) MIF/MAF – macrophage interactions. Biochemical characterization of a putative glycolipid receptor for MIF and the existence and properties of two distinct MIFs. In *Mononuclear Phagocytes*. Part I,, *Functional Aspects* (edited by VAN FURTH, R.), pp. 527–535. The Hague, Boston, London: Nijhoff.

DE PETRIS, S. (1974) Inhjbition and reversal of capping by cytochalasin B, vinblastine and colchicine. *Nature, Lond.* 250, 54–6.

DONALDSON, K., DAVIS, J. M. G. & JAMES, K. (1982) Characteristics of peritoneal macrophages induced by asbestos. *Environmental Res.* (in press).

GOLDSTEIN, B. D., HAMBURGER, S. J., FALK; G. W. & AMOROSO, M. A. (1977) Effect of ozone and nitrogen dioxide on the agglutination of rat alveolar macrophage by Concanavalin A. *Life Sci.* 21, 1637–44.

HADLEY, J. G., GARDNER, D. C., COFFIN, D. L. & MENZEL, D. B. (1977) Enhanced binding of autologous red cells to the macrophage plasma membrane as a sensitive indicator of pollutant damage. In *Pulmonary Macrophage and Epithelial Cells* (edited by SAUNDERS, C. L., SCHNEIDER, P. P., DAGLE, G. E. and REGAN, H. A.), pp. 66–77. New York: ERDA.

HAMMOND, M. E. & DVORAK, H. F. (1972) Antigen induced stimulation of glucosamine incorporation by guinea pig peritoneal macrophages in delayed hypersensitivity. *J. exp. Med.* 136, 1518–32.

HOMMA, Y., ONOZAKI, K., HASHIMOTO, T., MIURA, K., NAGAO, S. & TANAKA, A. (1981) Different effect of (L) fucose binding lectin on macrophage migration inhibition caused by guinea pig migration inhibition factor and synthetic muramyl dipeptide. *Int. Arch. Allerg. appl. Immun.* 65, 27–33.

HOPPER, K. E., WOOD, P. C. & NELSON, D. S. (1979) Macrophage heterogeneity. *Vox. Sang.* 36, 257–74.

HUNTER, W. M. (1973) Radioimmunoassay. In *Handbook of Experimental Immunology* (edited by WEIR, D. M.), pp. 17.1–17.36. Edinburgh: Blackwell.

JAMES, K., McBRIDE, B. & STUART, A. E. (1977) (editors) *The Macrophage and Cancer*. Edinburgh: James, McBride and Stuart.

LIPSCOMB, M. F., TOEWS, G. B., LYONS, C. R. & UHE, J. W. (1981) Antigen presentation by guinea pig alveolar macrophages. *J. Immun.* 126, 286–91.

LOOR, F. & ROELANTS, G. E. (1974) The dynamic state of the macrophage plasma membrane. Attachment and fate of immunoglobulin, antigen and lectins. *Eur. J. Immun.* 4, 649–60.

LUTTON, J. D. (1973) The effect of phagocytosis and spreading on macrophage surface receptors for Concanavalin A. *J. Cell Biol.* 56, 611–7.

MANTOVANI, B. (1981) Phagocytosis of immune complexes mediated by IgM and C_3 receptors by macrophages from mice treated with glycogen. *J. Immun.* 226, 127–30.

MARQUES, E. (1978) Binding of Concanavalin A by normal and transformed cells and by their trypsin released surface components. *Expl Cell Res.* 115, 143–9.

MILLER, K. (1978) The effects of asbestos on macrophages, *CRC Crit. Rev. Toxicol.* 5, 319–54.

MORAHAN, P. S. (1980) Macrophage nomenclature; where are we going? *J. Reticuloendothel. Soc.* 27, 223–45.

NABARRA, B., CAVELIER, J. F. & DIMITRIU, A. (1978) Scanning electron microscope studies of activated macrophages in the mouse. *J. Reticuloendothel. Soc.* 24 489–98.

NATHAN, C. F. & ROOT, R. K. (1977) Hydrogen peroxide release from mouse peritoneal macrophages. *J. Exp. Med.* 246, 1648–62.

OGMUNDSDOTTIR, H. & WEIR, I. M. (1980) Mechanisms of macrophage activation. *Clin. exp. Immun.* 40, 233–4.

OLIVER, J. M. & BERLIN, R. D. (1975) Macrophage membranes. In *Immunobiology of the Macrophage* (edited by NELSON, D. S.), pp. 000–000. London, New York: Academic Press.

OLIVER, J. M., BERLIN, R. D., BAEHNER, R. L. & BOXER, L. A. (1977) Mechanism of microtubule disassembly *in vivo*; studies on normal and chronic granulomatous disease leucocytes. *Br. J. Haemat.* 37, 311–22.

PICK, E. & WILNER, I. (1979) Cytoskeletal control of Concanavalin A receptor mobility in peritoneal macrophages. *Expl Cell Res.* 118, 151–8.

RABINOWITZ, M. & HAMBURG, S. I. (1980) Macrophage activation by interferon or interferon inducers. In *Mononuclear Phagocytes*, Part I, *Functional Aspects* (edited by VAN FURTH, R.), pp. 1515–1536. The Hague, Boston, London: Nijhoff.

RHODES, J. (1975) Macrophage heterogeneity in receptor activity – the activation of macrophage Fc receptor function *in vivo* and *in vitro*. *J. Immun.* 114, 976–81.

ROSENTHAL, A. S., BLAKE, J. T., ELLNER, J. J., GREINEDAR, I. K. & LIPSKY, P. E. (1976) Macrophage function in antigen recognition by T lymphocytes. In *Immunobiology of the Macrophage* (edited by NELSON, D. S.), pp 131–160. New York, London: Academic Press.

UNANUE, E. R. & ROSENTHAL, A. S. (1980) *Macrophage Regulation of Immunity*. New York, London: Academic Press.

WALTERS, M. N. & PAPADIMITRIOU, J. M. (1978) Phagocytosis – a review. *CRC Crit. Rev. Toxicol.* 5, 377–421.

WILLIAMS, D. A., BOXER, L. A., OLIVER, J. M. & BAEHNER, R. L. (1977) Cytoskeletal regulation of Concanavalin A capping in pulmonary alveolar macrophages. *Nature, Lond.* 267, 255–7.

WILLIAMS, D. A., LESSARD, J., BOXER, L. A., REIN, D. & BAEHNER, R. L. (1979) Metabolic and structural requirements for Concanavalin A capping in phagocytic cells. *Proc. Soc. exp. Biol. Med.* 162, 34–7.

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I made a mistake in the manuscript with regard to this reference it should be:- Mononuclear Phagocytes. Functional Aspects Part II (edited by van Furth R) pp 1515 - 1536 etc

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Characteristics of Peritoneal Macrophages Induced by Asbestos Injection

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The effect of single intraperitoneal injections of UICC crocidolite, UICC chrysotile, and a latex control particulate on the induced murine peritoneal macrophage population was measured. Spreading, Fc receptor avidity, and phagocytosis were measured 3, 18, and 70 days after injection. When activation of the induced macrophage populations was found at all three times with asbestos, but not with latex, experiments were undertaken to determine whether the asbestos-activated macrophages had attained the full tumoricidally activated state. An *in vitro* assay measuring macrophage cytotoxicity to tumor cells revealed that the tumoricidal potential of asbestos-activated macrophages was low at 3 and 5 days and negligible by 35 days after injection. An *in vivo* assay measuring the effect of asbestos on tumor growth generally supported the contention that asbestos-activated macrophages were not tumoricidal, although one dose of UICC chrysotile did produce a small, significant reduction in growth of a concomitant tumor. It was concluded that a single intraperitoneal asbestos injection in mice induces activated macrophages which do not become fully tumoricidal.

INTRODUCTION

The pathology associated with asbestos exposure in man and animals is well documented and includes lung fibrosis, mesothelioma, and carcinoma of the lung (Harrington *et al.*, 1975; Becklake, 1976; Selikoff and Lee, 1978). Additionally, disturbances of the immune system have been detected in asbestos workers (Kagan *et al.*, 1977; Haslam *et al.*, 1978; Lange, 1980), although the place of these disturbances in the etiology of asbestos-related disease is speculative.

The alveolar macrophage, due to its intimate interaction with foreign particulates during their clearance from the lung, has been intensively studied as to its possible role in these diseases (Miller, 1978). The toxic effect of asbestos, particularly chrysotile, on normal macrophages in culture has been compared to the action of silica and a model of asbestos-induced lung fibrosis has been evolved whereby macrophages, damaged and dying following asbestos phagocytosis, release hydrolytic enzymes and cell contents leading to stimulation of fibrogenesis (Allison, 1971). This model was subsequently modified when it was shown that normal macrophages in culture could be stimulated by low levels of asbestos to selectively release hydrolytic enzymes without cell death (Davies *et al.*, 1974). Evidence that this stimulatory, rather than toxic, effect of asbestos was operative *in vivo* was obtained by Miller and Kagan (1976), who found activation of the alveolar macrophages of rats inhaling crocidolite asbestos. Bateman *et al.* (1980),

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using an *in vivo* diffusion chamber containg macrophages and chrysotile asbestos at a dose found by Davies *et al.* (1974) to be stimulatory but not toxic, found evidence that a diffusible fibrogenic factor was released from the asbestos-treated macrophages.

Miller (1978) emphasized the likely importance of the secretory products of asbestos-activated macrophages in chronic asbestos inflammation, while the multiple roles of the macrophage in both humoral and cellular immunity (see reviews in Nelson, 1976; and Unanue and Rosenthal, 1980) suggest that immunological factors may also be important. The macrophage is also important in tumor development (see papers in James *et al.*, 1977) and so the role of the asbestos-activated macrophage in asbestos tumorigenesis requires investigation.

Although peritoneal macrophages differ in some properties from alveolar macrophages (Walker, 1976), they have been used as convenient alternatives in studies of the pathogenesis of asbestos-induced fibrosis (Miller, 1978). Injection of dust into the peritoneal cavity has also been used frequently as an alternative to inhalation since fibrosis and mesothelioma can also be produced in the peritoneal cavity using suitable dusts (Selikoff and Lee, 1978).

The experiments reported here were undertaken as part of a study into the effects of asbestos on cell membranes. The effect of a single intraperitoneal injection of asbestos on spreading activity, Fc receptor avidity, and phagocytic activity was measured since these properties of the macrophage membrane are known to be altered on activation (Cohn, 1978; Rhodes, 1975). The asbestos-induced peritoneal macrophages were found to be activated by the criteria of enhanced spreading and Fc receptor avidity. In view of the suggestion (Cohn, 1978; Hibbs *et al.*, 1980) that activation is a sequential development of properties leading to the fully microbicidal tumoricidal macrophage and since in the present study activation was present as measured by the criteria of spreading and Fc receptor avidity, the tumoricidal potential of the asbestos-activated macrophages was measured using an *in vitro* assay. An *in vivo* assay measuring the effect of intraperitoneal asbestos on the growth of a concomitant subcutaneous (s/c) tumor was also used.

MATERIALS AND METHODS

General. Male CBA/Ca mice 10–14 weeks of age at the time of injection were used throughout. Groups of three mice were injected intraperitoneally with (a) 2.5 mg of crocidolite asbestos (UICC sample) in 1 ml of Dulbecco A; (b) 2.5 mg of chrysotile asbestos (UICC sample) in 1 ml of Dulbecco A; (c) 1 ml of 1% latex (Bacto-latex, 0.81-μm diam, Difco); and (d) 1 ml of Dulbecco A. Groups of animals were killed 3, 18, and 70 days after treatment. Two separate experiments were carried out and the results were pooled.

Preparation of peritoneal exudate macrophages. Mice were killed by ether overdose at the selected time points and the peritoneal cavity was washed out with 6 ml of RPMI 16/40 medium containing 10 U/ml Heparin. The peritoneal exudate cells (PEC), which were always >95% viable by trypan blue exclusion, were washed and adjusted to 1×10^6/ml in RPMI containing 10% fetal calf serum (FCS). One hundred microliters of this suspension were dropped onto 6 × 22-mm coverslips which were incubated on racks for 1 hr at 37°C to allow for attachment of

macrophages. Before they were used in any assay these coverslip cultures were washed vigorously in a large volume of phosphate-buffered saline (PBS) to remove nonadherent cells. Cultures for the spreading assay were stained immediately after the 1-hr incubation and the other assays were carried out with 4 hr.

Spreading assay. After exactly 1 hr of incubation washed coverslip cultures were placed in May Grünwald solution and a May Grünwald/Giemsa stain was carried out. The number of spread macrophages in a total of 200 cells was expressed as a percentage.

Fc receptor assay. Sheep red blood cells (SRBCs) (Oxoid) sensitized with a 1:10 or 1:1000 solution of anti-SRBC serum, or 1:10 normal rabbit serum, were prepared at 1% in RPMI. One hundred microliters of sensitized SRBCs were added to coverslip cultures and left for 30 min at room temperature. Coverslips were washed carefully and inverted on a drop of toluidine blue on a glass slide. More than two red cells attached to a macrophage constituted a rosette and the number of rosette-forming cells in 200 cells was expressed as a percentage. Nonspecific rosettes demonstrated with normal rabbit serum-coated SRBC were present as a background of 7.6 ± 2.2% (SD).

Phagocytosis assay. One hundred microliters of 1% latex were added to coverslip cultures and incubated at 37°C for 1.5 hr. Coverslips were then vigorously washed in PBS to remove free latex and the coverslip was inverted on a drop of toluidine blue on a glass slide. The number of cells which had phagocytosed latex in a total of 200 cells was expressed as a percentage.

In vitro assay of macrophage cytotoxicity to tumor cells. The method used was essentially the radioactive release assay described by Mantovani *et al.* (1980) with the modification of Poste (1979), where the radioactivity associated with the target cells at the end of the assay period was measured. Cultured CCH_1 fibrosarcoma target cells (James *et al.*, 1979) were labeled by culturing in RPMI with 10% FCS, antibiotics, and 30 μCi [^{3}H]thymidine. Cells maintained in this way had a specific activity of 10,000−30,000 cpm/10^6 cells and spontaneous loss of activity over the 2 days of the assay was around 10%/day. To obtain the macrophage effector cells, PEC were collected 3, 5, and 35[1] after saline or asbestos injection and 10^6, 5×10^5, 10^5, and 5×10^4 cells were seeded into triplicate wells of Falcon 24-well plates. Wells were washed after 1 hr to remove nonadherent cells. 10^5 washed and labeled target cells were then added to each well yielding ratios of initial PEC to target cells of 10:1, 5:1, 1:1, and 0.5:1. After 2 days of culture 0.2% ethylene diamine tetraacetic acid (EDTA) was added to detach the tumor cells and their removal was further aided by pipetting. The EDTA-released tumor cells were harvested onto glass fiber paper in a cell harvester (Skatron) and the amount of [^{3}H]thymidine associated with the tumor cells was assessed by scintillation

[1] Previous data have revealed that induction of the tumoricidal response in macrophages following stimulation is an early and rapid event ~~[e.g. Flexman and Shellam, *Brit. J. Cancer* 42(1), 41–51), 3 days for ip *C. parvum* (Poste, 1979) and 4 days for subcutaneous glass coverslip.]~~ We therefore expected any tumoricidal macrophage induction by asbestos possibly by Day 3 and certainly by Day 5. Induction of the tumoricidal state suddenly, a long time after initial stimulus, has no precedent known to us; choice of the 70-day time point instead of the 35-day one would therefore have provided no additional information.

[for example 3 days for ip *C. parvum* (Flexman and Shellam 1980 Br. J. Cancer 42 41-51) and 4 days for subcutaneous glass coverslip (Poste 1979).]

counting. As a positive control 5-day *Corynebacterium parvum* (1.4 mg heat killed, Wellcome Laboratories)-induced macrophages were included in all experiments. Since asbestos was always injected in saline suspension and saline alone was found to induce a small amount of tumor cell cytotoxic activity in the peritoneal macrophage population, specific toxicity to tumor cells was calculated as follows for each ratio:

$$\% \text{ specific toxicity to tumor cells} = 100 - \left[\frac{\text{tumor cell bound counts following culture with asbestos-induced macrophages}}{\text{tumor cell bound counts following culture with saline-induced macrophages}} \times 100 \right].$$

Three separate experiments were carried out for each time point.

The effect of intraperitoneal asbestos injection on the growth of a concomitant subcutaneous tumor. As an adjunct to the *in vitro* assay of macrophage cytotoxicity to tumor cells an assay designed to detect any effects of asbestos on tumor growth *in vivo* was utilized. Various amounts of asbestos, saline, or *C. parvum* were injected intraperitoneally either 2 days prior to, or 3 days after, subcutaneous inoculation with 10^6 or 5×10^2 CCH_1 fibrosarcoma cells. Groups of ten mice were used for each condition and tumor growth was measured as the mean of the two major diameters of the tumor.

Analysis of results. Results were analyzed for significance using the *t* test.

RESULTS

The mean number of cells in the control peritoneal exudate was approximately 4×10^6 throughout and the latex-induced exudates never differed from this level (Fig. 1). The asbestos-induced exudates, however, contained approximately five times the control number of cells at Day 3, had fallen to control values at Day 18, and had risen again to approximately three times control levels by Day 70. Be-

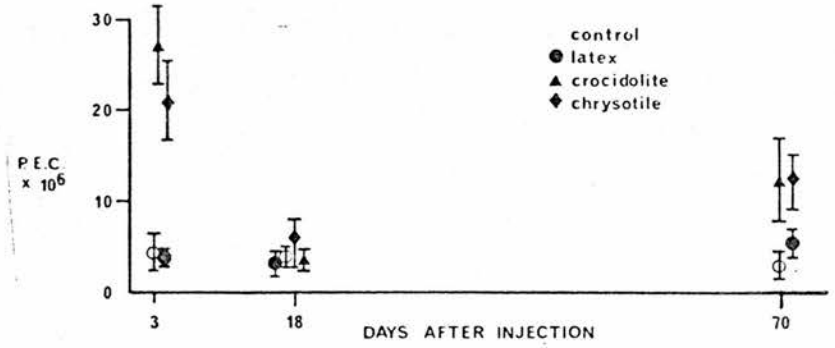

FIG. 1. Mean number of peritoneal exudate cells harvested from mice at various times after intraperitoneal injection of 1 ml saline (control), 1 ml 1% latex, 1 ml/2.5 mg UICC crocidolite or 1 ml/2.5 mg UICC chrysotile. Bars denote ± one standard deviation.

Please change all P values to $P < 0.002$. throughout

tween 50 and 70% of the cells in all exudates were macrophages by adherence difference; the remainder of the cells were polymorphs, which were increased at 3 days, and lymphocytes, with a few mast cells also present.

Both chrysotile and crocidolite injection resulted in the presence of activated macrophage populations in the peritoneal cavity at all three time points as judged by increased spreading (Fig. 2) and increased ability to form Fc rosettes with lightly sensitized SRBCs (Fig. 3). Spreading activity of asbestos-induced macrophages was maximal at 3 days, declining thereafter, but at all three time points the percentage spread was still significantly greater ($P = < 0.02$, see Fig. 2 for details) than that of saline-induced macrophages. Fc receptor avidity of asbestos-induced macrophages, demonstrated by increased ability to bind and form rosettes with lightly (1:1000 dilution of anti-SRBC) sensitized SRBC, remained similar at all three time points, being significantly greater ($P = < 0.05$, see Fig. 3 for details) than that of latex- or saline-induced macrophages. The latex-induced macrophages were never different from the saline-induced macrophages in any parameter except for the 3-day Fc receptor avidity which was significantly greater ($P = < 0.05$). The mean proportion of macrophages able to phagocytose latex was decreased on Day 3, but only significantly reduced ($P = < 0.05$) for crocidolite (Fig. 4). At succeeding time points, however, all macrophage populations had the same proportion, approximately 95%, of cells able to phagocytose latex. At Day 3, and to a lesser extent at Day 18, phagocytosed asbestos fibers were clearly visible inside crocidolite-induced macrophages and also inside chrysotile-induced macrophages, although chrysotile is less easily seen by light microscopy. The presence of such an ingested fiber load at Day 3 may have rendered some macrophages refractory to further phagocytosis during the latex phagocytosis assay.

The *in vitro* assay for macrophage cytotoxicity to tumor cells revealed that, compared to the *C. parvum*-elicited positive control macrophages, asbestos was relatively ineffective in inducing macrophage tumor cell cytotoxic activity (Fig. 5).

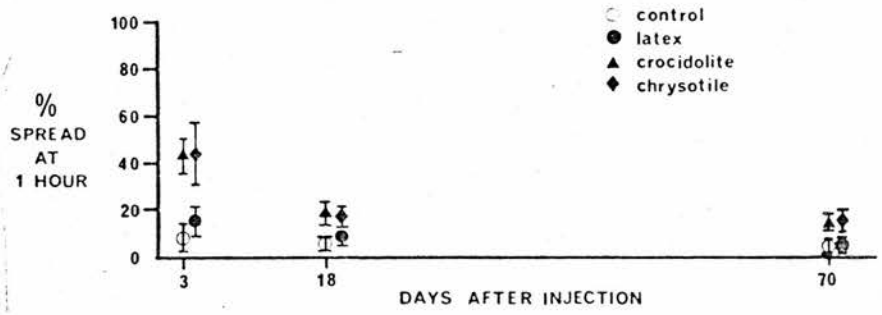

FIG. 2. Mean percentage of macrophages spread after plating onto glass coverslips and incubating for 1 hr at 37°C. Macrophages from PEC induced as in Fig. 1. Bars denote ± one standard deviation. At all three time points there was a statistically significant increase in the spreading of crocidolite- and chrysotile-induced macrophages compared to saline-induced macrophages. Levels of significance: 3-day crocidolite and chrysotile, $P = < 0.01$; 18-day crocidolite and chrysotile, $P = < 0.01$; 70-day crocidolite, $P = < 0.002$; 70-day chyrsotile, $P = < 0.02$.

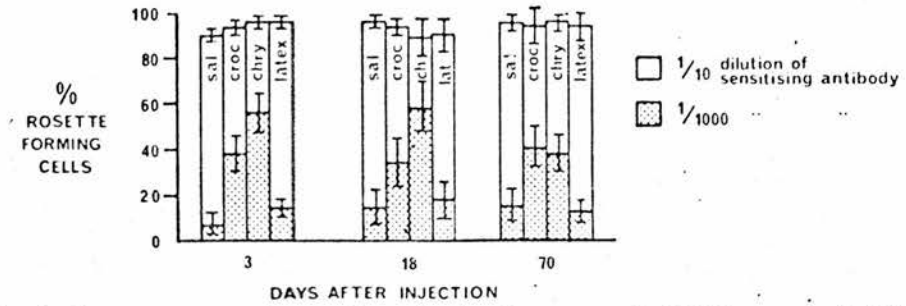

FIG. 3. Mean percentage of macrophages forming Fc rosettes with SRBCs coated with 1:10 and 1:100 dilutions of anti-SRBC serum. Macrophages from PEC induced as in Fig. 1. Bars denote ± one standard deviation. At all three time points crocidolite- and chrysotile-induced macrophages had a statistically significant increase in the percentage able to form rosettes with lightly sensitized SRBC compared to saline-induced macrophages. Levels of significance: 3-day crocidolite and chrysotile, $P = < 0.01$; 18-day crocidolite, $P = < 0.05$; 18-day chrysotile, $P = < 0.01$; 70-day crocidolite and chrysotile, $P = < 0.02$. At 3 days latex also produced a significant increase ($P = < 0.05$) in this parameter over saline-induced macrophages.

At 3 days, with chrysotile and crocidolite, macrophages had mean toxicities between 10 and 15%, while 5-day chrysotile-induced macrophages had attained a mean of 20% toxicity; 5-day crocidolite macrophages had only 5% mean toxicity. By 35 days the two asbestos types had very low cytotoxicities to the target cells. The highest toxicities obtained were always at the greatest effector: target ratio of 10:1. In this system 5-day *C. parvum*-induced macrophages produced a mean from all experiments of 27.4% toxicity at 5:1 and 54.0% toxicity at 10:1.

The low level of tumor cell cytotoxic activity induced by intraperitoneal asbestos was reflected in the results of the *in vivo* assay measuring the effect of intraperitoneal asbestos on the growth of a subcutaneous tumor (Table 1). Treatment with chrysotile 3 days after subcutaneous inoculation with 10^6 tumor cells was the only regimen which produced small but significant reductions in tumor size compared with saline-injected controls; this result was obtained in two separate experiments. The same regimen but with 2.5 mg or 20 mg chrysotile given

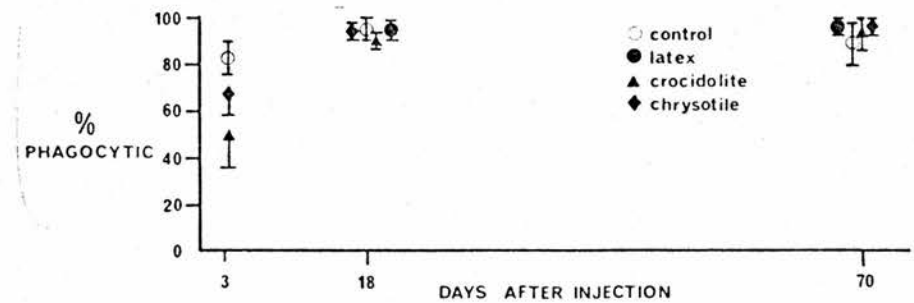

FIG. 4. Mean percentage of macrophages which had ingested latex particles following incubation for 1.5 hr in 1% latex. Macrophages from PEC induced as in Fig. 1. Bars denote ± one standard deviation. At 3 days crocidolite-induced macrophages had a significantly smaller ($P = < 0.05$) population able to phagocytose latex than did saline-induced macrophages.

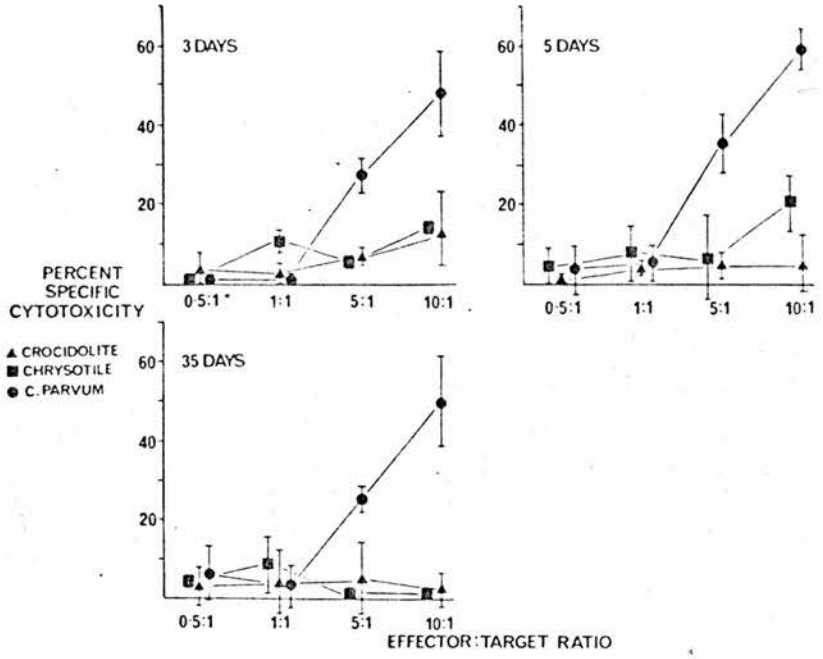

FIG. 5. Mean percentage specific cytotoxicity to tumor cells of macrophages induced by intraperitoneal injection of 2.5 mg UICC crocidolite, 2.5 mg UICC chrysotile, or 1.4 mg *C. parvum*. See text for calculation of specific cytotoxicity. Bars denote ± one standard deviation.

intraperitoneally did not produce significant reductions in tumor size. The use of 5 mg crocidolite in the same regimen also failed toproduce significantly smaller tumors. Decreasing the dose of inoculating tumor cells to 5×10^2 in the hope of emphasizing the 5-mg chrysotile effect resulted in abolition of the effect. Treating with 5 mg chrysotile 2 days before inoculation with 5×10^2 tumor cells, a regimen which greatly increased the efficacy of *C. parvum* in reducing tumor growth (Table 1), produced no significant reduction in tumor growth over that of saline-injected controls.

DISCUSSION

This study has shown that a single intraperitoneal injection of both chrysotile and crocidolite asbestos induces increased numbers of cells in the peritoneal exudate at 3 and 70 days, while the number of cells at 18 days was close to that of saline-injected controls. Latex injection never produced a cellular exudate greater in magnitude than that produced by injection of saline. The biphasic variation in numbers of peritoneal exudate cells with time following asbestos injection suggests that the cytotactic stimulus for recruitment of cells into the peritoneal cavity does not simply decay with time after the acute large-scale recruitment at Day 3 but is renewed between Days 18 and 70.

The macrophages present in the asbestos-induced exudate were activated at all three time points as measured by the criteria of increased Fc receptor avidity (Rhodes, 1975) and increased macrophage spreading (Cohn, 1978). Increased

TABLE 1

EFFECT OF VARIOUS DOSES OF INTRAPERITONEAL ASBESTOS, GIVEN EITHER BEFORE OR AFTER 10^6 OR 5×10^2 SUBCUTANEOUSLY ADMINISTERED CCH_1 FIBROSARCOMA CELLS, ON THE MEAN DIAMETER OF THE TUMOR

Number of tumor cells injected s/c on Day 0	Asbestos type and amount	Day of ip asbestos injection	Tumor size (mm ± SD)[a]	
			Asbestos group	Control group
10^6	2.5 mg chrysotile	+3	16.8 ± 1.2	17.6 ± 1.2
10^6	5 mg chrysotile	+3	13.8 ± 0.8	15.6 ± 1.2[b]
10^6	20 mg chrysotile	+3	14.5 ± 3.4	15.6 ± 3.3
5×10^2	5 mg chrysotile	+3	12.3 ± 2.1	13.1 ± 2.9
5×10^2	5 mg chrysotile	−2	13.2 ± 3.9	13.0 ± 3.8
10^6	5 mg crocidolite	+3	12.3 ± 0.7	13.6 ± 2.0
10^6	1.4 mg *C. parvum*	+3	10.1 ± 1.8	13.6 ± 2.0[b]
5×10^2	1.4 mg *C. parvum*	−2	2.8 ± 3.7	13.0 ± 3.8[c]

[a] For mice injected with 10^6 CCH_1 cells the size given is for Day 17; for mice injected with 5×10^2 CCH_1 cells the size given is for Day 31.

[b] Significant difference $P = < 0.002$.

[c] Significant difference $P = < 0.001$.

phagocytosis is also a marker of macrophage activation (Cohn, 1978), although decreases in phagocytosis with activation have also been reported (David and Remold, 1973). In the present study the 3-day crocidolite and chrysotile macrophage populations had a smaller percentage of macrophages which could phagocytose latex than did the 3-day saline-induced macrophages; only crocidolite macrophages, however, were significantly less. The 3-day saline-induced macrophages also had a mean phagocytic population which was smaller, but not significantly, than at subsequent time points. It must be emphasized that the assay used here measured the proportion of cells able to phagocytose latex and not the amount of latex phagocytosed by the total population. The macrophages induced by intraperitoneal injection of latex only once differed significantly from control values in any measurement, thus confirming that latex is not a macrophage-activating agent (Schnyder and Baggiolini, 1978).

The *in vitro* assay for macrophage cytotoxicity to tumor cells revealed a low level of tumor cell cytotoxicity in the 3- and 5-day asbestos macrophages and the virtual absence of such activity by 35 days. This low level of tumoricidal activity was present despite evidence of macrophage activation by spreading and Fc receptor avidity, suggesting that, in the murine peritoneal model, asbestos induces only partial activation in the major proportion of the activated population and that only a small proportion have obtained the fully activated state.

The *in vivo* assay measuring the effect of intraperitoneal asbestos on tumor growth produced conflicting, mostly negative, results. Chrysotile produced significant reductions in tumor size at 5 mg but not at 20 mg, while crocidolite at 5 mg did not produce significant reductions in tumor size. Adoption of a regimen which profoundly increased the ability of *C. parvum* to produce reductions in tumor size caused 5 mg chrysotile to lose its effect in reducing subcutaneous tumor growth.

These results are difficult to interpret, but one interpretation could be that the mechanism whereby 5 mg chrysotile ip reduces tumor size is different from the mechanism whereby *C. parvum* ip brings about the same effect.

A model of macrophage activation to the tumoricidal state has evolved, suggesting that there is a complex multistep pathway to the fully tumoricidal state involving serial intermediate stages obtained by differentiation signals (Russell *et al.*, 1978; North, 1978; Cohn, 1978; Hibbs *et al.*, 1980). Such studies have suggested that macrophages activated to a nontumoricidal state by sterile inflammatory irritants, BCG, or toxoplasma infection are in a condition where they can be raised to the fully tumoricidal state by endotoxin treatment or, as is more likely *in vivo*, by the lymphokine MAF (macrophage-activating factor) produced as a result of a cell-mediated immune response (Russell *et al.*, 1978; Hibbs *et al.*, 1980). A corollary of this model is that only newly recruited macrophages are capable of being activated to the tumoricidal state and indeed Poste (1979) has shown experimentally that only newly recruited inflammatory macrophages can have their tumoricidal function induced, or natural tumoricidal function augmented, by lymphokine treatment.

The results of the present study could be interpreted, therefore, as evidence that a single injection of asbestos provides the inflammatory stimulus, and a large influx of newly recruited macrophages into the peritoneal cavity was certainly observed at Day 3, but that levels of MAF were insufficient to induce the fully activated tumoricidal state at any of the time points. In this respect it is of interest that Miller *et al.* (1979) observed alveolar macrophage activation and manifestations of cellular immunity in the lungs of rats inhaling asbestos, although they did not measure lymphokine levels. It is possible, therefore, that the single injection, peritoneal model used in the present study is an unsuitable one for studying asbestos activation of macrophages due either to the use of the peritoneal site or to the single dose compared to the multiple dosing produced by inhalation. If, however, the model used here does result in a cellular immune response and MAF generation then the asbestos-induced macrophages could be rather insensitive to its effect in generating tumoricidally activated macrophages. Such a refractory state, if it existed, could be a contributory factor in asbestos carcinogenesis.

The results presented here for the tumor cell cytotoxicity of asbestos-activated macrophages are rather less than that for inflammatory macrophgaes generated using glass, another insoluble irritant which is also carcinogenic (Bischoff and Bryson, 1964). In this study Poste (1979) collected macrophages on subcutaneously implanted glass coverslips and demonstrated greater than 40% spontaneous toxicity to tumor cells for a limited period between 4 and 7 days.

In the present study no attempt was made to measure Ia antigen expression in the membrane of asbestos-elicited macrophages. Ia expression is an important parameter in the antigen-presenting function of macrophages and it has been shown to be inducible in peritoneal macrophages by ip injection of activating agents (Beller and Unanue, 1981). Complete characterization of the asbestos-activated macrophage and its role will require study of the kinetics of Ia expression and the antigen-presenting ability of these macrophages.

The present study has shown that a single intraperitoneal injection of asbestos

results in the induction of activated peritoneal macrophages in the short and long term, but in neither case is the fully activated, tumoricidal state attained. These results lend support to previous findings that asbestos can activate alveolar macrophages following inhalation (Miller and Kagan, 1976) and can activate peritoneal macrophages *in vitro* as shown by increased release of lysosomal enzymes in the absence of cell death (Davies *et al.*, 1974). They also confirm the findings of Hamilton (1980), who showed that intraperitoneal asbestos produced macrophage activation as judged by increased plasminogen activator secretion.

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REFERENCES

Allison, A. C. (1971). Lysosomes and the toxicity of particulate pollutants. *Arch. Intern. Med.* **128**, 131–139.

Bateman, E., Emerson, R., and Cole, P. (1980). The use of diffusion chambers to examine the biological effects of mineral dusts. *In* "The *in Vitro* Effects of Mineral Dusts" (R. C. Brown, I. P. Gormley, M. Chamberlain, and R. Davies, Eds.), pp. 289–296. Academic Press, New York/London.

Becklake, M. R. (1976). Asbestos-related diseases of the lung and other organs: Their epidemiology and implications for clinical practice. *Amer. Rev. Respir. Dis.* **114**, 187–227.

Beller, D. I., and Unanue, E. R. (1981). Regulation of macrophage populations. II. Synthesis and expression of Ia antigens by peritoneal exudate macrophages is a transient event. *J. Immunol.* **126**, 263–269.

Bischoff, F., and Bryson, G. (1964). Carcinogenesis through solid state surfaces. *Progr. Exp. Tumour Res.* **5**, 85–133.

Cohn, Z. A. (1978). The activation of mononuclear phagocytes: Fact, fancy and future. *J. Immunol.* **121**, 813–816.

David, J. R., and Remold, H. G. (1976). Macrophage activation by lymphocyte mediators and studies on the interaction of macrophage inhibitory factor (M. I. F.) with its target cell. *In* "Immunobiology of the Macrophage" (D. S. Nelson, Ed.), pp. 401–426. Academic Press, New York/London.

Davies, P., Allison, A., Ackerman, J., Butterfield, A., and Williams, S. (1974). Asbestos induces selective release of lysosomal enzymes from mononuclear phagocytes. *Nature (London)* **251**, 423–425.

Hamilton, J. A. (1980). Macrophage stimulation and the inflammatory response to asbestos. *Environ. Health Perspect.* **34**, 69–74.

Harfington, J. S., Allison, A. C., and Badami, D. V. (1975). Mineral fibres: Chemical, physicochemical and biological properties. *Advan. Pharmacol. Chemother.* **12**, 291–402.

Haslam, P. L., Lukoszek, A., Merchant, J. A., and Turner-Warwick, M. (1978). Lymphocyte responses to phytohaemagglutinin in patients with asbestosis and mesothelioma. Clin. Exp. Immunol. **31**, 178–188.

Hibbs, J. B., Weinberg, J. B., and Chapman, H. A. (1980). Modulation of the tumoricidal function of activated macrophages by bacterial endotoxin and mammalian macrophage activation factor(s). *In* "Macrophages and Lymphocytes: Nature Functions and Interactions, Part B" (M. R. Escobar and H. Friedman, Eds.), pp. 433–435. Plenum, New York.

James, K., McBride, B., and Stuart, A. (Eds.) (1977). "The Macrophage and Cancer." The Editors, Edinburgh.

James, K., Milne, I., Merriman, J., and McBride, W. H. (1979). Further studies in anti tumour responses induced by short-term pre-treatment with syngeneic tumour cells. *Brit. J. Cancer* **39**, 122–131.

Kagan, E., Solomon, A., Cochrane, J. C., Beissner, E. I., Gluckman, J., Rocks, P. H., and Webster, I. (1977). Immunological studies of patients with asbestosis, 1 and 2. *Clin. Exp. Immunol.* **28**, 261–275.

Lange, A. (1980). An epidemiological survey of immunological abnormalities in asbestos workers, 1 and 2. *Environ. Res.* **22**, 162–183.

Mantovani, A., Dean, J. H., Jerrels, T. R., and Herberman, R. B. (1980). Augmentation of tumouricidal activity of human monocytes and macrophages by lymphokines. *Int. J. Cancer* **25**, 691–699.

Miller, K. (1978). The effects of asbestos on macrophages. *CRC Crit. Rev. Toxicol.* **5**, 319–353.

Miller, K., and Kagan, E. (1976). The *in vivo* effects of asbestos on macrophage membrane structure and population characteristics of macrophages. A scanning electron microscope study. *J. Reticuloendothel. Soc.* **20**, 159–171.

Miller, K., Weintraub, Z., and Kagan, E. (1979). Manifestations of cellular immunity in the rat after prolonged asbestos inhalation. 1. Physical interactions between alveolar macrophages and splenic lymphotyces. *J. Immunol.* **123**, 1029–1038.

Nelson, D. S. (Ed.) (1976). "Immunobiology of the Macrophage." Academic Press, New York/London.

North, R. J. (1978). The concept of the activated macrophage. *J. Immunol.* **121**, 806–809.

Poste, G. (1979). The tumouricidal properties of inflammatory tissue macrophages and multinucleate giant cells. *Amer. J. Pathol.* **96**, 595–608.

Rhodes, J. (1975). Macrophage heterogeneity in receptor activity: The activation of macrophage Fc receptor function *in vivo* and *in vitro*. *J. Immunol.* **114**, 976–981.

Russell, S. W., Doe, W. F., and McIntosh, A. T. (1977). A non-cytolytic stage of macrophage activation in Moloney sarcomas. *In* "The Macrophage and Cancer" (K. James, B. McBride, and A. Stuart, Eds.), pp. 341–346. The Editors, Edinburgh.

Schnyder, J., and Baggiolini, M. (1978). The role of phagocytosis in the activation of macrophages. *J. Exp. Med.* **148**, 1449–1457.

Selikoff, I. J., and Lee, D. H. K. (1978). "Asbestos and Disease." Academic Press, New York/London.

Unanue, E. R., and Rosenthal, A. S. (1980). "Macrophage Regulation of Immunity." Academic Press, New York/London.

Walker, W. S. (1976). Functional heterogeneity of macrophages. *In* "Immunobiology of the Macrophage" (D. S. Nelson, Ed.), pp. 91–110. Academic Press, New York/London.